# *Bacillus licheniformis* specific DNA markers for identification and culture-independent monitoring

Maria Eduarda Marques de Almeida
Mestrado em Biologia Celular e Molecular
Departamento de Biologia
2014

**Orientador**
Fernando Tavares, Professor Doutor, Faculdade de Ciências, Universidade do Porto




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

“*Não sei o que acontecerá no futuro ao Homem, mas uma certeza eu tenho, nós vamos e elas (bactérias) ficam*.”

– Prof. Fernando Tavares

# Acknowledgements

The past year was a learning journey, in which the help and dedication of colleagues was precious to accomplish this work. Support of family and friends was also essential and will not be forgotten. To all of them, I express my deepest and sincere appreciation.

First, I would like to express my deepest and truly thanks to my supervisor, Prof. Fernando Tavares, for always pushing me to think further about my work and also for the constant guidance and support. I know that sometimes it was not easy for him, but I assure that his inspiring words will always be remembered in my mind. Foremost, he is a mentor to me.

I also would like to address a special thanks to Pedro Albuquerque that was always nearby to give me a word of guidance, when my (many, many,…) doubts arose. His endless patience to guide and support me and his countless advices to help me were priceless. Also, without all his help my work in the laboratory would have been much more complicated. So, Pedro, you are right when you say to me “és uma chata”.

A special thanks to Cláudia Serra for teaching me so many things with infinite patience, with always a smile in her face and a friendly word to guide me, even in her busiest moments. She was always ready to offer all the knowledge that I needed, without asking anything in return.

There are no words to thank these three persons: Prof. Fernando Tavares, Pedro Albuquerque and Cláudia Serra.

Also, I would like to thank Inês Guerreiro from NUTRIMU for providing the samples used in this work, for always being available to help me and for all her kindness.

I would like also to thank Prof. Aires Oliva Teles and Paula Enes for always being welcoming at the NUTRIMU. Also, thank you to Patricia Díaz-Rosales and Filipe Coutinho by guiding me throughout all qPCR experiment.

I am also grateful to Dr. Adriano O. Henriques from Microbial Development group at ITQB for the *Bacillus* strains that he kindly provided.

Very special thanks to Tiago Araújo who became an essential part of my life and knows me better than anyone (and did not run away). I know that not always has been easy to deal with me, but I am here also for him, through the best and the worst.

To my closest friends, I have to show them my gratitude for the help on clearing my mind for few but valuable moments. In particular to Joana Mendes, who kindly forced me to have dinner (and cooked for me) when I was passing through the busiest times. Also, she always had a motivating word for me.

My sincere thanks to Cristina Dias from MDE group for the company, sympathy and for giving me the sentence “vai correr tudo bem” in the most difficult moments.

Also, I would like to thank Maria João Fonseca at CIBIO for the invitation to present the project of this work.

Finally, I would never be here presenting this work without the help of my parents and brother. They have always been there for me, even in the moments that I had not been present for them. Thank you, mom, dad and little brother.

Also, I will dedicate this work to my dear grandfather who recently passed away. We are always thinking that life of the closed ones will ends in another day, but at any moment we can be surprised.

# Abstract

Worldwide consumption of fish per capita increased from 9.9 kg in the 1960s to 19.2 kg in 2012. With this increase in fish utilization, aquaculture emerged as an attractive alternative to intensive fishing and as a complementary strategy to capture fisheries. Consequently, aquaculture production recently surpassed capture fisheries, being responsible for nearly 50 percent of the world's food fish supply.

The increase in aquaculture production led to the challenge of a sustainable development in this field, with special emphasis placed on reducing the environmental impacts of this practice.

Among the fish produced in aquaculture, gilthead seabream is one of the success stories, since it showed a rapid and high adaptability to the intensive rearing systems used. However, gilthead seabream farming is also affected by the main problems associated with aquaculture farming: disease prevention and control.

As a prevention measure, antibiotics were commonly used as growth promoters. However, this practice has been banned in Europe, leading to an increased interest in more sustainable alternatives for disease prevention, such as probiotics, prebiotics and synbiotics.

*Bacillus subtilis* and *B. licheniformis* are the most used probiotics in aquaculture. Among these, *B. licheniformis* presents an ability to produce extracellular enzymes that are able to digest proteins and complex polysaccharides, conferring them a high potential to metabolize prebiotics.

Fructooligosaccharides (FOS) are a commonly used prebiotic in aquaculture and several microbial enzymes associated with its utilization have been reported to exist in known beneficial gut bacteria, namely *Lactobacillus* and *Bifidobacterium* species. On the other hand, the FOS effect in populations of *Bacillus* is poorly understood. To assess the effects of this relationship on the host, synbiotics approaches combining FOS with *Bacillus* strains reported several beneficial effects, such as increased disease resistance and improved growth.

Effects of probiotics, prebiotics or synbiotics are usually inferred through the effects observed on the host, namely growth, survival and immune response, or through

measurement of bacterial bioproducts. In order to screen the dynamics of *B. licheniformis* populations directly in seabream gut, the development of improved culture-independent detection methods is needed.

The present work aimed to establish quantitative real-time PCR (qPCR) as a routine method for bacterial quantification directly in environmental samples. To achieve this goal, specific DNA markers for *B. licheniformis* were selected using CUPID and Insignia bioinformatics tools. These utilities, combined with *in silico* genomic studies, have proved to be a reliable approach for selection of taxa-specific markers, since the DNA markers selected in this work were successfully applied for identification and detection of *B. licheniformis*.

To understand the dynamics of *B. licheniformis* populations in gilthead seabream gut, a qPCR based method, using the selected *B. licheniformis* specific markers, was developed taking into account several key steps for an accurate bacterial quantification.

Additionally, we also aimed to assess the ability of *B. licheniformis* to metabolize FOS within the gilthead seabream gut by feeding these fish with diets differently enriched with FOS. The results suggested that none of the diets were able to trigger the growth of *B. licheniformis* populations to values above the qPCR limit of quantification (LOQ), i.e. to allow reliable quantification. Consequently, determination of the ability of FOS to modulate *B. licheniformis* populations was not possible. Further optimization of the qPCR methodology is needed to reduce LOQ, allowing an accurate quantification of *B. licheniformis* population in environmental samples.

Regardless the current limitations, the novel DNA markers characterized in this work were shown to be reliable tools for culture-independent identification and detection of *B. licheniformis*, allowing to monitor these bacteria in environmental samples, which we believe to be a solid contribution to better understand their ecology.

Keywords: *Bacillus licheniformis*, taxa-specific DNA markers, CUPID, Insignia, qPCR

# Resumo

O consumo mundial *per capita* de peixe aumentou de 9.9 kg nos anos 60 para 19.2 kg no ano de 2012. Com este aumento, a aquacultura surgiu como uma alternativa à pesca intensiva e como uma estratégia complementar para as pescas de captura, sendo, neste momento, responsável por quase 50 % do fornecimento mundial de peixe. O aumento da produção de peixe em aquacultura levou à necessidade do seu desenvolvimento sustentável, com especial ênfase para a redução dos impactos ambientais desta actividade.

Das diferentes espécies de peixes produzidos em aquacultura, a dourada revelou ser um dos casos de sucesso, pois mostrou uma rápida adaptação aos sistemas de criação intensiva. Contudo, a produção deste peixe nestes sistemas é afectada pelo maior problema associado à aquacultura: prevenção e controlo de doenças.

Os antibióticos foram tradicionalmente usados como promotores do crescimento. Porém, esta prática foi proibida na Europa, conduzindo a um maior interesse em alternativas mais sustentáveis para a prevenção de doenças, como os probióticos, prebióticos e sinbióticos.

Os probióticos mais usados em aquacultura são *B. subtilis* e *B. licheniformis*. Entre estes, o *B. licheniformis* apresenta uma grande capacidade para produzir enzimas extracelulares que são capazes de digerir proteínas e polissacarídeos, conferindo-lhes, desta forma, um grande potencial para metabolizar prebióticos.

Os frutooligossacarídeos (FOS) são prebióticos frequentemente utilizados em aquacultura e várias enzimas microbianas associadas com a sua metabolização foram descritas como sendo sintetizadas por conhecidos probióticos, nomeadamente as espécies de *Lactobacillus* e *Bifidobacterium*. No entanto, o efeito dos FOS nas populações de *Bacillus* continua pouco estudado. Para compreender os efeitos da interacção destes dois agentes no hospedeiro, os FOS combinados com estirpes de *Bacillus* (sinbióticos) foram usados na alimentação de peixes, resultando em vários efeitos benéficos, designadamente uma resistência a doenças melhorada e um maior crescimento do hospedeiro.

Os efeitos dos probióticos, prebióticos e sinbióticos são normalmente inferidos através de efeitos observados no hospedeiro, mais especificamente, através do

crescimento, da sobrevivência e da resposta imunitária destes, ou, por outro lado, através da medição de bioprodutos bacterianos. De modo a compreender a dinâmica de populações de *B. licheniformis* no intestino da dourada, é necessário o desenvolvimento e melhoramento de métodos de detecção independentes do isolamento em meio de cultura.

O presente trabalho teve como objectivo estabelecer qPCR como um método de rotina para quantificação directa de bactérias em amostras ambientais. Para atingir este objectivo, foram seleccionados marcadores de DNA específicos para *B. licheniformis*, usando as ferramentas bioinformáticas CUPID e Insignia. Estes recursos combinados com estudos genómicos realizados *in silico*, provaram ser uma metodologia adequada para a selecção de marcadores específicos, na medida em que os marcadores de DNA seleccionados foram aplicados com sucesso para a identificação e detecção de *B. licheniformis*.

Para determinar a dinâmica das populações de *B. licheniformis* no intestino da dourada e tendo em conta vários passos-chave para uma adequada quantificação bacteriana, foi desenvolvido um método de qPCR, utilizando os marcadores específicos de *B. licheniformis*.

Adicionalmente, tivemos também como objectivo determinar a capacidade de *B. licheniformis* para metabolizar FOS no interior do intestino da dourada, através da alimentação destes peixes com dietas diferencialmente suplementadas com FOS. Os resultados sugerem que nenhuma das dietas aplicadas foi capaz de favorecer a multiplicação de *B. licheniformis* para valores acima do limite de quantificação (LOQ) do qPCR, i.e. que permitissem uma quantificação consistente. Em consequência, a capacidade de *B. licheniformis* para metabolizar FOS não foi possível de determinar. A optimização do qPCR poderá levar à redução do LOQ desta técnica, permitido uma correcta quantificação da população de *B. licheniformis* nas amostras ambientais.

Independentemente das presentes limitações, os novos marcadores de DNA caracterizados neste trabalho mostraram ser ferramentas adequadas para a identificação e detecção de *B. licheniformis* usando métodos independentes do isolamento em meio de cultura. Esta abordagem permitiu monitorizar estas bactérias em amostras ambientais, o que a nosso ver é uma contribuição sólida para um melhor entendimento da ecologia de *B. licheniformis*.

Palavras-chave: *Bacillus licheniformis*, marcadores de DNA, CUPID, Insignia, qPCR.

# Table of Contents

# List of Tables

# List of Figures

# List of Attachments

# List of Abbreviations

**ATCC –** American Type Culture Collection

**ATP –** Adenosine triphospate

**AXOS –** Arabinoxylooligosaccharides

**BCCM –** Belgian Coordinated Collections of Microorganisms

**BGSC –** Bacillus Genetic Stock Center

**BLAST –** Basic Local Alignment Search Tool

**CAI –** Codon Adaptation Index

**CFU –** Colony-forming unit

**CIBIO –** Centro de Investigação em Biodiversidade e Recursos Genéticos

**CIIMAR –** Centro Interdisciplinar de Investigação Marinha e Ambiental

**Cq(s) –** Quantification cycle(s)

**CUPID –** Core and Unique Protein Identification

**DGRM –** Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos

**DNA –** Deoxyribonucleic acid

**dNTP –** Deoxynucleoside triphosphate

**EC –** Enzyme Commission number

**eCAI –** Expected Codon Adaptation Index

**EU –** European Union

**FAO –** Food and Agriculture Organization

**FCUP –** Faculdade de Ciências da Universidade do Porto

**FOS –** Fructooligosaccharides

**GC content –** Percentage of guanine or cytosine bases

**GOS –** Galactooligosaccharides

**HK(s) –** Housekeeping gene(s)

**IMO –** Isomaltooligosaccharides

**IPC –** Internal Positive Control

**IS –** Insertion elements

**LB –** Luria-Bertani medium

**LOQ –** Limit of quantification

**MDE –** Microbial Diversity and Evolution group

**MLST –** Multilocus sequence typing

**MOS –** Mannanoligosaccharides

**MRS –** Man, Rogosa and Sharp medium

**NA –** Nutrient Agar

**NCBI –** National Center for Biotechnology Information

**NTC –** Non-Template Control

**NUTRIMU –** Fish Nutrition and Immunobiology group

**ORF(s) –** Open Reading Frame(s)

**PCR –** Polymerase Chain Reaction

**qPCR –** Quantitative Real-Time Polymerase Chain Reaction

**RDP –** Ribossomal Database Project

**rRNA –** Ribosomal ribonucleic acid

**SCFAS –** Short-chain fatty acids

**scFOS –** Short-chain fructooligosaccharides

**SMS –** Sequence Manipulation Suite

**SPC(s) –** Sample Processing Control(s)

**tRNAs –** Transfer ribonucleic acids

**UniProt –** Universal protein resource

**VBNC –** Viable but nonculturable bacteria

**WGS –** Whole-genome shotgun contigs

**WHO –** World Health Organization

**XOS –** Xylooligosaccharides

# 1. Introduction

The steady increase of world population is directly tied to a higher exploitation of all kind of food sources. In what concerns the average of fish utilization, worldwide consumption per capita increased from 9.9 kg in the 1960s to 19.2 kg in 2012 (Fig. 1) (FAO, 2014a). For many decades, capture fisheries were the main strategy adopted for fish supply. With the increase in population and consequently the increase in fish utilization, it was clear that capture fisheries were not able to supply all the required fish, unless an intensive fishing strategy was applied. This measure is however associated with several potential negative environmental impacts, namely changes within ecosystems biodiversity (FAO, 2014b).

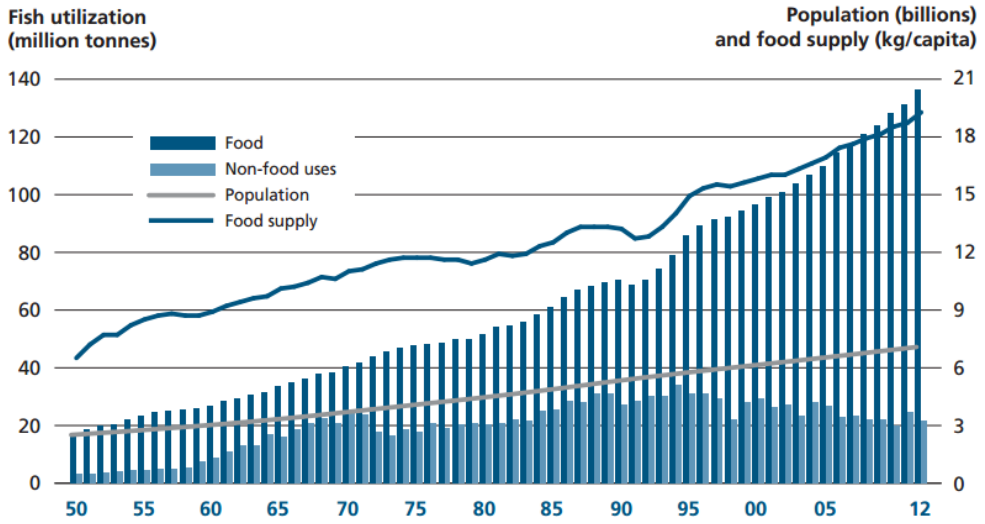



**Fig. 1 –** World fish utilization and supply (FAO, 2014a).

Around the 1970s, aquaculture, which is defined as the farming of aquatic organisms either in coastal or inland areas, including interventions in the rearing process to enhance production (FAO, 2014c), emerged as an alternative to intensive fishing and as a complementary strategy to capture fisheries. Presently, aquaculture production is considered the fastest growing food-production sector (Fig. 2), accounting for nearly 50 percent of the world's food fish supply (FAO, 2014d).

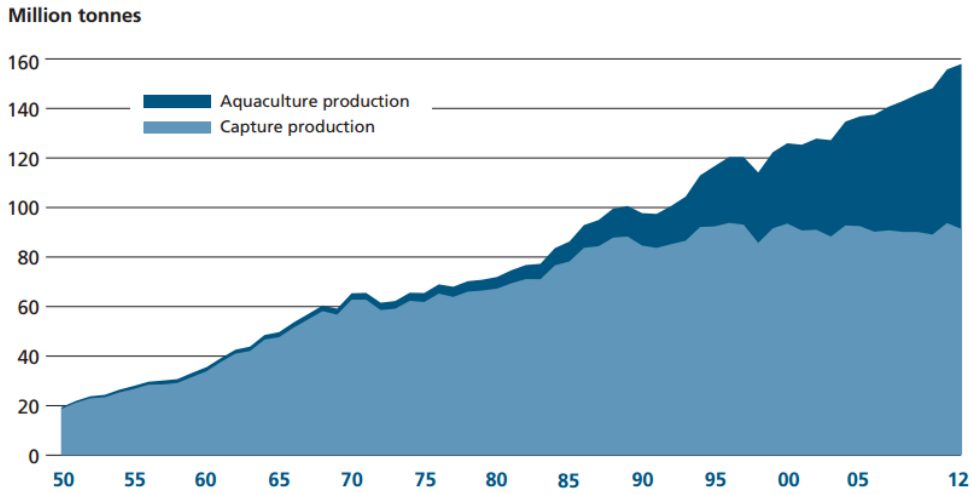



**Fig. 2 –** World capture fisheries and aquaculture production (FAO, 2014a).

The increase in aquaculture production led to the challenge of a sustainable development in this field. The major concerns of past, present and future decades are to reduce the environmental impacts of aquaculture and also avoid impacts on aquaculture arising from non-aquaculture activities (FAO, 2014e). Consequently, new measures were suggested and implemented as integrated aquaculture-agriculture farming systems, which have been applied, for instance, in Asia, where certain species of fish are used to fight golden snail rice pest. This approach allows simultaneously to boost rice yields and to harvest fish (FAO, 2014f).

Other concerns under study are the introduction of new species into aquaculture systems, the development of new production technologies, implementation of efficient diets and disease control. Also, the aquaculture licensing process is in most cases the biggest impediment to the expansion of this sector. In order to simplify this step, responsible organizations are pre-defining production areas, where species and respective densities are already defined (Framian, 2009).

Several organizations are involved in the regulation and improvement of aquaculture practices, as the Food and Agriculture Organization of the United Nations (FAO) that works at an international level (FAO, 2014g). In Europe, Aquaculture is also regulated by the European Commission (European Commission, 2014) and in Portugal, the government entity that is mainly responsible for aquaculture regulation is the Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos (DGRM).

According to the DGRM (2014), the main aquaculture species produced in Portugal are rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), clam (*Ruditapes decussatus*), carpetshell (*Venerupis pullastra*), cockle (*Cerastoderma edule*), cuttlefish

(*Sepia officinalis*), eel (*Anguilla anguilla*), sole (*Solea* spp.), pod razor (*Pharus legumen*), sword razor (*Ensis* spp.), mussel (*Mytilus* spp.), Portuguese cupped oyster (*Crassostrea* spp.), cupped oyster (*Ostrea* spp.), turbot (*Psetta maxima*), seabass (*Dicentrarchus labrax*), white seabream (*Diplodus sargus*), mullet (*Mugil* spp./*Liza* spp.) and gilthead seabream (*Sparus aurata*). Among these, gilthead seabream (or only seabream) represents one of the success stories in aquaculture. Initially, seabream was extensively cultured in its natural habitat until the 1980s, when intensive rearing systems were developed and seabream quickly showed a high adaptability to these systems (FAO, 2014h).

Gilthead seabream is a benthopelagic fish of both marine and brackishwater environments, such as coastal lagoons and estuarine areas (FAO, 2014h, 2014i; FishBase, 2014). The seabream worldwide distribution (Fig. 3) is restricted to Mediterranean Sea, Atlantic Ocean from British Isles to Cape Verde, and rarely in Black Sea (FAO, 2014i, 2014j; FishBase, 2014).

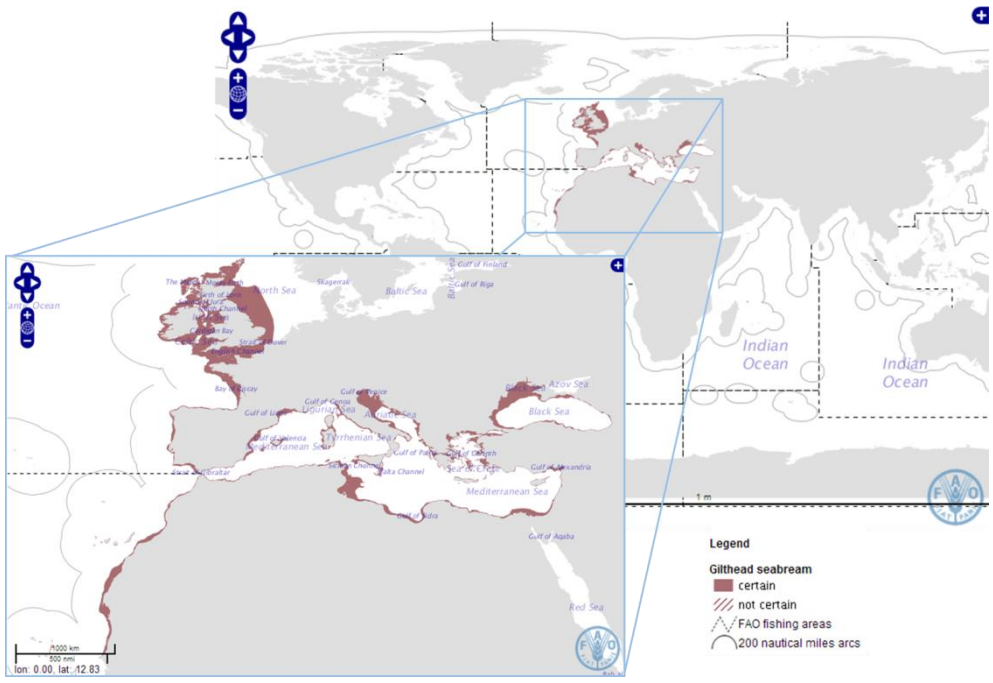



**Fig. 3 –** Gilthead seabream world distribution (FAO, 2014j).

The main problems associated with aquaculture farming are disease prevention and control, and seabream farming is not an exception (FAO, 2014h). Intensive fish farming systems, which resulted from enhanced need of fish supply, have been responsible for the emergence of several bacterial diseases mainly due to the physiologically stressed and/or immune suppressed state of intensively farmed fish. Under these conditions,

opportunistic pathogens are able to colonize fish, causing disease and, consequently, leading to a higher use of antibiotics (Alderman and Hastings, 1998; Barton and Ywama, 1991; Naylor and Burke, 2005; Walker, 2004), not only for therapeutic reasons, but also as a metaphylactic or prophylactic measure (Romero et al., 2012).

Use of antimicrobial drugs in aquaculture exerts selective pressure on pathogens, as well as on environmental bacteria and beneficial microbiota, selecting for antibiotic resistant bacteria. Moreover, fish absorption of antibiotics is low and 70 to 80% of administered antibiotics are released to the aquatic environment (Martinsen and Horsberg, 1995; Samuelsen, 2006; Smith and Samuelsen, 1996), where they have been associated with an increased number of antibiotic resistant bacteria (Huys et al., 2000; Miranda and Zemelman, 2002a, 2002b; Schmidt et al., 2000, 2001a, 2001b). Also, the excessive use of antibiotics led to the presence of residual levels of antibiotics in food products from aquaculture, which may induce changes in consumers' microbiota (Angulo et al., 2004; Goldburg et al., 2001; Grave et al., 1996, 1999).

Antibiotic resistance genes can be located in mobile genetic elements, such as plasmids and transposable elements. Therefore, genes conferring antibiotic resistance can be transferred between bacteria through lateral DNA transfer, namely by transformation (uptake of foreign environmental DNA), conjugation (uptake of genes through cell-to-cell contact) and transduction (uptake of genes through an infection with viral DNA). Interestingly, transduction may play an important role in aquaculture as a trait of gene transfer, once high concentrations of bacteria are present in seawater and marine sediment, where viruses are also abundant (Fuhrman, 1999). Additionally, gene transfer was reported not only between aquatic bacteria but also between aquatic and terrestrial bacteria (Agersø and Guardabassi, 2005; Casas et al., 2005; Fuhrman, 1999), representing a major threat to human health, since transfer of antibiotic resistance genes to human pathogens was already reported (Angulo, 2000; Weber et al., 1994).

Recently, the use of antibiotics as growth promoters has been banned in Europe (EU Regulation no. 1831/2003), which led to an increased interest in sustainable alternatives. Phage therapy, growth and virulence inhibition, green water, probiotics (Defoirdt et al., 2007), prebiotics and synbiotics (Gibson and Roberfroid, 1995) are all disease preventive measures that were targeted for study as alternatives to the prophylactic use of antibiotics.

According to FAO/WHO recommendations, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit

on the host" (Araya et al., 2002). Historically, the first report of what we known today as probiotics is attributed to Metchnikoff (1907), which noted that ingestion of fermented milk products containing microorganisms was responsible for health beneficial effects.

*Bacillus* strains have been used for decades as probiotics. An example is a probiotic containing *Bacillus clausii* spores named Enterogermina®, which was registered in 1958 in Italy (Cutting, 2011). *Bacillus* spp. are gram-positive, aerobic and endospore-forming microorganisms whose spores are able to survive extreme environmental conditions. From a probiotic point of view, production of spores represent a major advantage over other non-spore formers probiotics, since this trait confers a longer storage life (Cutting, 2011) and a cheaper cost of probiotic production (Wang et al., 2008). Also, spores survives passage through the upper gastrointestinal tract with low pH (Barbosa et al., 2005; Spinosa et al., 2000), which allows bacteria to reach the small intestine where they exert their probiotic effect. However, selection of *Bacillus* strains for probiotics must be a meticulous and rigorous process, since some strains used as probiotics have also been reported as carriers of multidrug resistance and toxin genes (Duc et al., 2004; Hoa et al., 2000).

In aquaculture, *Bacillus* probiotics have been associated with competitive exclusion events, i.e. the decrease of pathogens caused by the increasing of beneficial bacteria, in white shrimp (Li et al., 2007), stimulation of seabream immunesystem (Salinas et al., 2005, 2008), improved water quality by decreasing the pathogenic population in water (Dalmin et al., 2001; Decamp et al., 2008; Moriarty, 1998; Vaseeharan and Ramasamy, 2003) and increased survival of black tiger shrimp (Rengpipat et al., 1998, 2003). These bacteria have been also reported as promoters of seabream larvae growth, which showed increased body weight and standard length. These later observations have been related with the release of digestive enzymes by *Bacillus* strains (Avella et al., 2010), which may lead to an improved digestion by the host (Bagheri et al., 2008; Ghosh et al., 2002; Nagano and To, 1999; Ziaei-Nejad et al., 2006) or to an additional provision of essential nutrients (Verschuere et al., 2000).

*B. subtilis* and *B. licheniformis* are the most used probiotics in aquaculture (Moriarty, 2003). Concerning *B. licheniformis*, when administered with other *Bacillus* strains, namely *B. subtilis* and *Bacillus pumilus*, an increased growth and immune resistance was observed in rainbow trout (Bagheri et al., 2008; Raida et al., 2003). An enhanced immune response was also detected in white shrimp culture, when *B. licheniformis* was the only administered probiotic (Li et al., 2007).

*B. licheniformis*, which is one of the better defined *Bacillus* species, is an ubiquitous, facultatively anaerobic and endospore-forming bacterium. *B. licheniformis* is also saprophytic, producing extracellular proteases and other enzymes that are able to digest complex polysaccharides (Claus and Berkeley, 1986). The enzyme production ability makes these bacteria an interesting target for prebiotics, which have been defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon" (Gibson and Roberfroid, 1995).

Increased interest in prebiotics emerged due to limitations of probiotics application, namely high cost, possible environmental impacts and regulatory issues, as well as the difficult maintenance of a constant level of probiotics in fish feeds and the decreasing viability of most probiotic strains during preparation and storage (Dimitroglou et al., 2011; Ringø et al., 2010).

The mechanism of action of prebiotics, which are not digested by the host, consists in a stimulation of activity and growth of beneficial gut bacteria, leading to an increased production of short-chain fatty acids (SCFAs) that consequently decreases pH. A low pH may promote a higher growth of probiotic bacteria and also suppress undesirable bacteria (Blaut, 2002). Additionally, Blaut (2002) have hypothesized that SCFAs may play an important role for the host by leading to an optimal functioning of the intestinal epithelium and higher absorption of several important cations, such as $Ca^{2+}$, $Mg^{2+}$ and $Fe^{2+}$.

Several prebiotics have already been applied in aquaculture practices, namely inulin, GroBiotic®-A, mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), fructooligosaccharides (FOS) and short-chain fructooligosaccharides (scFOS) (Ringø et al., 2010). A well-known and widely used prebiotic is FOS, which have several microbial enzymes associated with its microbial utilization, specifically fructosidase EC 3.2.1.26 (Barrangou et al., 2003; Goh et al., 2006, 2007), inulinase EC 3.2.1.7 (Mckellar and Modler, 1989; Xiao et al., 1989), levanase EC 3.2.1.65 (Menéndez et al., 2002), fructofuranosidase EC 3.2.1.26 (Rossi et al., 2005; Saulnier et al., 2007), fructanase EC3.2.1.80 (Hartemink et al., 1995) and levan biohydrolase EC 3.2.1.64 (Saito et al., 2000; Song et al., 2002). This apparent diversity of enzymes masks the fact that these are all functionally related, since they are involved in the hydrolysis of β-D-fructosidic linkages, releasing fructose. Also, they share the conserved motif H-x(2)-P-x(4)-[LIVM]-N-D-P-N-G. Therefore, Naumoff (2001)

suggested that these enzymes should be considered as members of the same β-fructosidase superfamily.

FOS digestion has been associated with bacterial growth and activity of *Lactobacillus* and *Bifidobacterium* strains (Buddington et al., 1996; Gibson et al., 1995; Williams et al., 1994), providing selective advantages to these beneficial gut bacteria, particularly by leading to the suppress of undesirable bacteria (Buddington et al., 2002). Nevertheless, the influence of FOS on gut native *Bacillus* strains remains poorly understood. Mahious et al. (2006) reported that Raftilose P95, which is a mixture of inulin and FOS, was able to increase weaning turbot growth, as well as the gut bacterial diversity and the emergence of *Bacillus* spp. as cultivable bacteria. These authors suggested that microbiota diversity and *Bacillus* spp. predominance might be responsible for the increased growth of the fish.

Furthermore, recent studies used *Bacillus* strains combined with FOS, which is denominated as a synbiotic approach i.e. synergistic combinations of probiotics and prebiotics (de Vrese and Schrezenmeir, 2008), to improve aquaculture production. Zhang et al. (2010) reported improved disease resistance through an enhanced immunity of sea cucumber when *B. subtilis* was administered with FOS. Sun and co-workers (2011) also provided *B. subtilis* and FOS to sea cucumber and observed an improved immunity response and growth. In triangular bream, administration of *B. licheniformis* along with FOS led to an increased disease resistance, as well as to an enhanced innate immunity and antioxidant capability (Zhang et al., 2013).

Synbiotic approaches have a great potential not only in aquaculture but also in livestock and human health, as a paradigm to conciliate a specific prebiotic with the most suitable probiotic. This will increase probiotic survival (Collins and Gibson, 1999) and persistence in the gut (Rastall and Maitin, 2002), resulting in advantages for the host, which are related to the type of prebiotics and probiotics administered (Collins and Gibson, 1999).

Effects of probiotics, prebiotics or synbiotics are usually inferred through the effects observed on the host, namely growth, survival and immune response, or through measurement of bacterial bioproducts as SFCAs and enzymes. When a direct study of colonization is carried out, the employed methods are the same as the ones used for bacterial detection (Balcázar et al., 2006). Traditionally, bacterial screening is performed through culture, serological, immunological and histological methods. Recently, molecular techniques, such as immunohistochemical tests, restriction enzyme digestion, probe hybridization, polymerase chain reaction (PCR) have been

recurrently applied for bacterial detection (Balcázar et al., 2006; Cunningham, 2002). All molecular approaches mentioned are able to detect the microbes present in a sample without bacterial culture, which is a major advantage since only a very small percentage of microorganisms are cultivable using the currently available culture media (Amann, 2000; Amann et al., 1995). Even though these methods are reliable tools for detection of bacteria, they are not able to quantify the amount of each taxon in a sample. Bacterial quantification is useful to understand microbial dynamics and modulation ability, when different treatments are applied as in the case of probiotics, prebiotics or synbiotics administration. On this scope, qPCR has been used for direct bacterial quantification using DNA extracted from environmental or clinical samples, including faecal samples to study the gut microbiota. The majority of these studies have been focused in humans (Centanni et al., 2013; Furet et al., 2009; Haarman and Knol, 2005; Jost et al., 2012, 2014; Larsen et al., 2010; Mariat et al., 2009; Menard et al., 2008), however when another model of study is considered the information is scarce. Also, most of these studies have used primers for amplification of 16S rRNA gene, which can be a bias due to the existence of multiple copies of this gene in a genome (Lee et al., 2008), high similarity between sequences from closely related strains and intragenomic variability (Michon et al., 2010). Currently, there are several bioinformatics tools that allow to select novel taxa-specific DNA markers, which can help to overcome this limitation (Albuquerque et al., 2009), such as CUPID (Core and Unique Protein Identification) (Mazumder et al., 2005) and Insignia (Phillippy et al., 2009), which provide taxa-specific proteins and taxa-specific DNA regions, respectively.

Specific DNA regions have been designated as DNA signatures, which, as proposed by Phillippy and collaborators (2007), are defined as “nucleotide sequences that can be used to detect the presence of an organism and to distinguish that organism from all other species” or as taxa-specific DNA markers. Recently, Albuquerque et al. (2011, 2012a, 2012b) showed that DNA signatures obtained with CUPID and Insignia are a reliable tool for bacterial detection. Therefore, specific DNA markers can be used as targets for qPCR, conferring several advantages over approaches targeting 16S rRNA, since the DNA signatures obtained can be present as single-copy in the genomes.

Presently, studies targeting bacterial quantification in fish microbiota are not yet reported. In fish farming, bacterial quantification using qPCR can be a powerful tool to assess the effectiveness of different treatments aimed to improve aquaculture

production quality, through the analysis of probiotics dynamics in fish gut microflora, such as *Bacillus* spp.

The present work aimed at the development of improved culture-independent methods able to screen the dynamics of *B. licheniformis* strains in seabream gut. For this purpose, an *in silico* analysis was carried out to identify novel taxa-specific DNA markers for *B. licheniformis*, which allow identification, detection and quantification of these strains.

The selected markers were designed in order to directly monitor *B. licheniformis* strains in gut samples using traditional, multiplex and quantitative PCR, allowing to surpass the bias usually associated with culture dependent approaches, such as inability to detect viable but nonculturable microorganisms (VBNC), low specificity and detection resolution. Importantly, this work aimed to establish qPCR as a reliable culture-independent method for bacterial quantification directly in environmental samples.

Beyond the optimization of a method to trace and quantify *B. licheniformis* in the gut, we aimed to assess the ability of *B. licheniformis* to metabolize FOS within the seabream gut by feeding these fish with diets differently enriched with FOS and, consequently, assessing the putative prebiotic activity of these dietary components on this bacterium i.e. the FOS ability to modulate *B. licheniformis* populations.

# 2. Material and Methods

## 2.1. Bacterial strains and DNA extraction

The majority of bacterial strains used in this work are from the Microbial Diversity and Evolution (MDE; CIBIO) and Fish Nutrition and Immunobiology (NUTRIMU; FCUP/CIIMAR) bacterial collections. These strains were isolated from gut of different fish (turbot, gilthead seabream and European seabass) in different experiences, which are summarized in Table 1. Briefly, approximately 300 mg of each faecal sample was suspended in 2 mL of peptone water, vortexed thoroughly and left for debris sedimentation. Faecal suspensions from turbot and seabream were 10-fold serially diluted in peptone water and 100 µL from each dilution was plated on Man, Rogosa and Sharp (MRS; Liofilchem, Italy) or Nutrient Agar (NA; Liofilchem) in the case of turbot samples, or only in NA (Liofilchem) in the case of seabream samples. Plates were incubated at 25 ºC and 30 ºC, respectively. To select for the bacterial population of spore formers such as *Bacillus* spp., faecal suspensions from European seabass were initially diluted 1:1 in ethanol and peptone water. Ethanol treated suspensions were agitated for 45 min (Sample treatment E – Table 1), while the peptone water suspensions were heat treated at 65 ºC for 30 min (Sample treatment H – Table 1). After treatments, both suspensions were 10-fold diluted in peptone water. From each dilution, 100 µL were plated onto NA (Liofilchem) and incubated at 30 ºC. All plates were incubated from 5 to 7 days.

*B. licheniformis* 9945A, *Bacillus subtilis* 168, *Bacillus pumilus* ATCC_7061 and *Bacillus megaterium* ATCC_19213 from *Bacillus* Genetic Stock Center (BGSC) bacterial collection, as well as *Bacillus sonorensis* LMG_21636, *Bacillus amyloliquefaciens* LMG_9814 and *Bacillus atrophaeus* LMG_16797 from Belgian Coordinated Collections of Microorganisms/LMG (BCCM/LMG) bacterial collection, were also used in this work.

All strains were grown overnight on Luria-Bertani Medium (LB; AppliChem, Germany) at 37 ºC. DNA from these cultures was extracted using the EZNA Bacterial DNA Purification Kit (Omega Bio-Tek, USA), following manufacturer's instructions, and

DNA quantification was carried out using Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay (Invitrogen, USA).

**Table 1 -** Bacterial isolates from MDE and NUTRIMU groups collections used in this work.

| Isolate | [a]Sample Treatment | Culture conditions for isolation | Host fish species |
|---|---|---|---|
| FI1 | n/a | MRS, 25 ºC | Turbot (*Scophthalmus maximus*) |
| FI2 | | | |
| FI3 | | | |
| FI11 | | NA, 25 ºC | |
| FI34 | n/a | NA, 30 ºC | Gilthead seabream (*Sparus aurata*) |
| FI35 | | | |
| FI38 | | | |
| FI39 | | | |
| FI40 | | | |
| FI42 | | | |
| FI44 | | | |
| FI46 | | | |
| FI47 | | | |
| FI94 | H | NA, 37 ºC | European seabass (*Dicentrarchus labrax*) |
| FI105 | H | | |
| FI120 | E | | |
| FI132 | H | | |
| FI136 | H | | |
| FI139 | E | | |
| FI141 | E | | |
| FI144 | H | | |
| FI152 | H | | |
| FI157 | E | | |
| FI159 | E | | |
| FI242 | E | | |
| FI268 | E | | |
| FI282 | E | | |

[a] Sample treatment: n/a – not applied; E – ethanol treatment; H – heat treatment.

### 2.2. Identification of bacterial isolates

Bacterial isolates obtained from fish gut were identified by 16S rRNA gene sequencing. PCR amplification using primers 27F and 1492R (Lane, 1991) was carried out in 20 µL PCR reactions containing 1 x DreamTaq Buffer (Thermo Scientific,

Germany), 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Thermo Scientific), 0.2 µM of each primer (STAB Vida, Portugal), 1 U of DreamTaq DNA Polymerase (Thermo Scientific) and 25 ng of DNA template. The PCR program consisted of 5 min initial denaturing at 95 ºC, 35 cycles of 30 s denaturing at 95 ºC, 30 s annealing at 55 ºC and 90 s extension at 72º C; and 10 min final extension at 72 ºC. PCR products were separated on 1 % agarose (Invitrogen) gels stained with GelRed (Biotium, USA). Gel electrophoresis was carried out at 80 V until the Orange G front is within 1 cm of the end of the gel.

Amplicons were purified from agarose gels or from PCR reactions using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, United Kingdom) and sent for sequencing at STAB Vida. Isolates were presumptively identified to the species level using BLAST analysis and the Ribosomal Database Project (RDP).

### 2.3. *In silico* selection of DNA markers

Taxa-specific regions for *B. licheniformis* were retrieved according to two flowcharts for selection of specific DNA markers, which were adapted from Albuquerque and collaborators (2012b) and represented in Fig. 4 (Flowcharts 1 and 2).

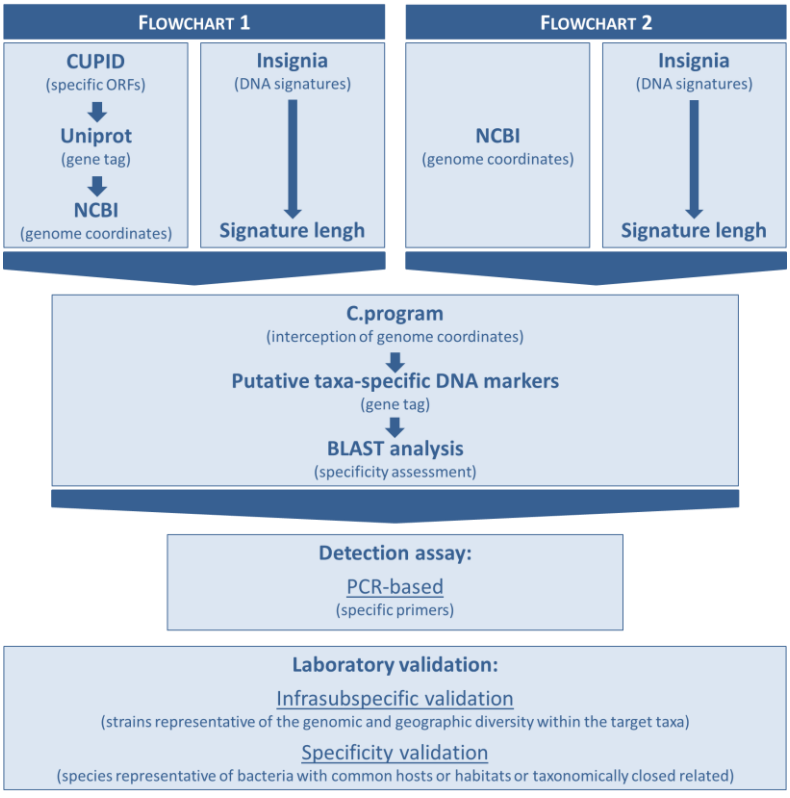



**Fig. 4 –** Flowcharts used for selection of novel taxa-specific DNA markers (adapted from Albuquerque et al. (2012b)).

Following Flowchart 1, the online databases CUPID (Mazumder et al., 2005) and Insignia (Phillippy et al., 2009) were used to respectively list the specific proteins and DNA signatures of *B. licheniformis* ATCC_14580, which was the strain used as reference. The outputs of both databases were overlapped using a C+.program (Albuquerque et al., 2012b).

Flowchart 2 was followed in parallel due to the low number of strains available for analysis in CUPID. In this approach, only the primary outputs from Insignia were accounted for analysis using *B. licheniformis* DSM_13 as the reference strain. In order to consider only putative coding regions, the output from Insignia was overlapped with the complete protein list of strain DSM_13, obtained from NCBI. In both approaches, signature length was set to a minimum of 99 bp in Insignia.

The most promising DNA signatures were selected through a robust BLAST (Basic Local Alignment Search Tool) analysis, using the nucleotide collection (nr/nt) and whole-genome shotgun contigs (wgs) databases (Altschul et al., 1990). BLAST thresholds were applied according to Albuquerque et al. (2012b) to guarantee the selection of significant blast hits. Primer design for the specific regions was carried out using the Vector NTI software (Invitrogen) and the predicted amplicons were again submitted to a BLAST analysis to ensure the specificity of the selected markers.

### 2.4. *In silico* analyses of DNA markers

To assess the putative stability and origin of the selected DNA regions, the location of each marker was determined in relation to several genomic mobilization-related features. Using Geneious R7 v7.1.7 (Biomatters, New Zealand), the circular chromosome of *B. licheniformis* ATCC_14580 was used to pinpoint elements associated with genomic mobility, namely phage related ORFs, insertion elements (IS), recombinases, integrases, transposases and tRNAs (Albuquerque et al., 2012a). The codon adaptation index (CAI), the expected CAI (eCAI) and the GC content of each ORF containing the selected markers were calculated using the CAIcaI sever (Puigbò et al., 2008). For comparison, these parameters were also calculated for six housekeeping genes (HKs) used for Multilocus Sequence Typing (MLST) of *B. licheniformis*: *adk*, *ccpA*, *recF*, *rpoB*, *spo0A* and *sucC* (Madslien et al., 2012).

Synteny plots comparing the genome of *B. licheniformis* type strain ATCC_14580 and two other fully sequenced *B. licheniformis* strains (DSM_13 and 9945A) were obtained using SynMap (Lyons et al., 2008). High resolution synteny analysis of the

regions containing the selected markers was carried out using the GEvo tool (Lyons and Freeling, 2008).

### 2.5. Experimental validation of selected markers

The specificity and stability of selected DNA markers was experimentally assessed by PCR, which was prepared as mentioned in section 2.2., using an annealing temperature of 59 ºC. The bacterial collection strain *B. licheniformis* 9945A and *B. licheniformis* strains previously isolated from fish (FI1, FI2, FI3 and FI11) were used as positive controls. Closely related strains, namely *Bacillus sonorensis* LMG_21636, *Bacillus subtilis* 168, *Bacillus amyloliquefaciens* LMG_9814, *Bacillus atrophaeus* LMG_16797, *Bacillus pumilus* ATCC_7061 and *Bacillus megaterium* ATCC_19213 were used as negative controls.

PCR products were separated on 2 % agarose (Invitrogen) gels stained with GelRed (Biotium). Gel electrophoresis was carried out as described in section 2.2. and amplicons were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions. To confirm the identity of the markers, the purified amplicons were sequenced at STAB Vida and the obtained sequences were aligned using Geneious R7.

### 2.6. **Feeding Trial** (in collaboration with NUTRIMU group)

#### 2.6.1. Diets

Four isolipid (18 % lipid) and isonitrogenous (45 % protein) diets were formulated in order to meet seabream nutritional requirements. At a ratio of 50:50, plant ingredients (soybean and wheat meal; corn and wheat gluten) and fish meal were used as the main protein sources, and fish oil was used as the lipid source. The experimental diets were differently enriched with short-chain fructooligosaccharides (scFOS; PROFEED Maxflow, France): diet D0 with 0 % (control diet), D0.1 with 0.1 %, D0.25 with 0.25 % and D0.5 with 0.5 % scFOS.

All ingredients were mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, USA), using a 2.0 mm die. Pellets were dried in an oven at 40 ºC during 48 h and stored in airtight bags until use (Guerreiro, 2013). Ingredients and proximate composition of diets are presented in Table 2.

**Table 2 -** Ingredients and proximate composition of the experimental diets (Guerreiro, unpublished work).

| | Diets | | | |
|---|---|---|---|---|
| | **D0** | **D0.1** | **D0.25** | **D0.5** |
| **Ingredients (% dry weight)** | | | | |
| Fish meal[1] | 31.4 | 31.4 | 31.4 | 31.4 |
| Corn gluten[2] | 5.0 | 5.0 | 5.0 | 5.0 |
| Wheat gluten[3] | 5.0 | 5.0 | 5.0 | 5.0 |
| Wheat meal[4] | 15.2 | 15.2 | 15.2 | 15.2 |
| Soy meal[5] | 25.0 | 25.0 | 25.0 | 25.0 |
| scFOS[6] | -- | 0.1 | 0.25 | 0.5 |
| Cellulose[7] | 0.5 | 0.4 | 0.25 | -- |
| Cod liver oil | 13.7 | 13.7 | 13.7 | 13.7 |
| Bicalcium phosphate[8] | 0.7 | 0.7 | 0.7 | 0.7 |
| Vitamin mix[9] | 1.0 | 1.0 | 1.0 | 1.0 |
| Mineral mix[10] | 1.0 | 1.0 | 1.0 | 1.0 |
| Choline chloride (50 %) | 0.5 | 0.5 | 0.5 | 0.5 |
| Binder (Aquacube)[11] | 1.0 | 1.0 | 1.0 | 1.0 |
| **Proximate analysis (% dry weight)** | | | | |
| Dry matter (%) | 87.4 | 87.3 | 89.1 | 88.5 |
| Crude protein | 45.8 | 46.6 | 45.7 | 46.6 |
| Crude fat | 18.7 | 18.0 | 18.0 | 18.3 |
| Ash | 9.3 | 9.1 | 9.2 | 9.3 |
| Starch | 10.8 | 10.5 | 11.4 | 11.1 |

[1] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A. Perú (CP: 71.7 % DM; CF: 9.5 % DM).
[2] Sorgal, S.A. Ovar, Portugal (CP: 72.2 % DM; CF: 2.0 % DM).
[3] Sorgal, S.A. Ovar, Portugal (CP: 84.4 % DM; CF: 1.8 % DM).
[4] Sorgal, S.A. Ovar, Portugal (CP: 14.1 % DM; CF: 3.2 % DM).
[5] Sorgal, S.A. Ovar, Portugal (CP: 50.2 % DM; CF: 2.4 % DM).
[6] PROFEED Maxflow "Fructo-Oligosaccharides" (Jefo, France).
[7] Sigma-Aldrich, Portugal.
[8] Premix, Portugal (Calcium: 24 %; Total phosphorus: 18 %).
[9] Vitamins (mg $kg^{-1}$ diet): retinol acetate, 18000 (IU $kg^{-1}$ diet); cholecalciferol, 2000 (IU $kg^{-1}$diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[10] Minerals (mg $kg^{-1}$diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite. 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g $kg^{-1}$diet); potassium chloride, 1.15 (g $kg^{-1}$diet); sodium chloride, 0.40 (g $kg^{-1}$diet).
[11] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).

2.6.2. Growth Trial

Gilthead seabream juveniles were obtained from a commercial fish farm (Maresa, S.A., Spain), kept in quarantine for one month and transferred to the experimental systems 15 days before the beginning of the trial, to allow fish adaptation to the experimental conditions. During those periods, fish were fed with a commercial diet containing 50 % protein and 12 % lipids.

The trial was carried out in a recirculating water system comprising 12 cylindrical fiberglass tanks of 100 L water capacity, at the experimental facilities of the Marine Zoology Station, Porto University, Portugal. A continuous flow of filtered seawater (2.5 – 3.5 L $min^{-1}$) with 35 ± 1 g $L^{-1}$ salinity was used to supply tanks, which were thermo-regulated to 25.0 ± 0.6 ºC. Water dissolved oxygen levels were maintained near saturation (7 mg $L^{-1}$) using aeration.

Twenty two gilthead seabream, with an initial mean body weight of 32.0 ± 0.01 g, were distributed to each tank. Diets were randomly attributed to triplicate groups of fish. The trial lasted eight weeks, during which fish were hand-fed, until apparent visual satiation, twice a day, six days a week. At the end of the trial, fish were sacrificed for collection, under aseptic conditions, of mucosa and digesta samples to assess the autochthonous and allochthonous microbiota, respectively. Samples collected from each tank were pooled into one sample to overtake inter-fish variation.

The entire experiment was executed by accredited scientists, according FELASA category C recommendations and to the European Union directive 2010/63/EU on the protection of animals used for scientific purposes.

## 2.7. DNA extraction from faecal samples

DNA extraction from faecal samples was performed according to Pitcher et al. (1989) with some modifications. Briefly, approximately 300 mg of intestinal content were ressuspended in 1 mL of TE buffer and centrifuged at 13000 g for 5 min. The supernatant was discarded and the pellet was ressuspended in 200 µL of TE containing 50 mg/mL of lysozyme and incubated for 30 min at 37 ºC. Cell lysis was carried out by adding 500 µL GES reagent (Pitcher et al., 1989). The suspension was gently mixed, 250 µL of ammonium acetate (7.5 M) were added, mixed thoroughly and the samples were incubated on ice for 10 min. Phenol-chloroform extraction was performed by adding 500 µL phenol-chloroform-isoamyl alcohol (25:24:1), centrifuging 10 min at 13000 g and retrieving the aqueous phase, to which 500 µL of chloroform-

isoamyl alcohol (24:1) were added. The mixture was centrifuged for 10 min at 13000 g and the aqueous phase was recovered. DNA was precipitated with 0.6 volumes of isopropanol, followed by a 10 min centrifugation at 13000 g. The supernatant was discarded and the pellet was washed with cold 80 % ethanol and dried at room temperature. DNA was ressuspended in 100 µL DEPC-treated and sterile filtered water (Sigma-Aldrich, Switzerland).

### 2.8. *B. licheniformis* detection in digesta samples

To screen the presence of *B. licheniformis* specific markers in seabream gut, DNA extracted from digesta samples was used in PCR reactions containing 1 x DreamTaq Buffer (Thermo Scientific), 0.2 mM of each dNTP (Thermo Scientific), 0.2 µM of each primer (STAB Vida), 1 U of DreamTaq DNA Polymerase (Thermo Scientific) and 2 µL of digesta DNA, in a 20 µL volume. PCR conditions were the same as mentioned in section 2.5., using a different annealing temperature of 53 ºC.

PCR products were separated on 2 % agarose (Invitrogen) gels stained with GelRed (Biotium). Gel electrophoresis was carried out as described in section 2.2.

### *2.9.* Multiplex PCR for identification of *B. licheniformis*

A multiplex PCR using the four *B. licheniformis* specific markers was tested in a 20 µL reaction with 1 x DreamTaq Buffer (Thermo Scientific), 0.2 mM of each dNTP (Thermo Scientific), 0.2 µM of each primer (STAB Vida), 1.5 U of DreamTaq DNA Polymerase (Thermo Scientific) and 25 ng of DNA template. PCR conditions applied were similar to those mentioned in section 2.5., with the exception for the annealing temperature that was set at 65 º.

PCR products were separated on 8 % polyacrylamide (Bio-Rad, USA) gels, for which electrophoresis was carried out at 25 mA/gel until the bromophenol blue front reached the bottom of the gel. These gels were stained with a GelRed (Biotium) solution.

### 2.10. qPCR for *B. licheniformis* quantification in digesta samples

In order to establish a qPCR experiment to quantify *B. licheniformis* in faecal samples several levels of screening were addressed, which are summarized in Fig. 5.

BL8A (247 bp) and BL18A (216 bp) markers were inserted into pGEM-T using pGEM®-T Easy Vector System I (Promega, USA), according to manufacturers' instructions, and cloned into *Escherichia coli* DH5α competent cells (New England BioLabs, USA) using the calcium chloride method. Plasmid DNA from ampicillin resistant colonies containing each vector-insert combination was extracted using GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, USA), and submitted to a digestion with NotI-HF (New England BioLabs) to confirm markers insertion.

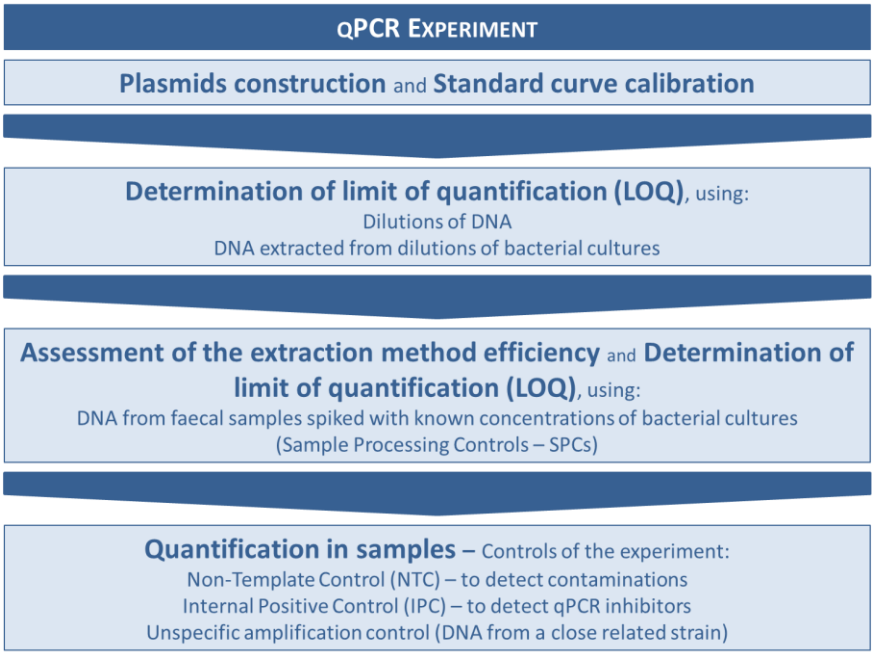



**Fig. 5 –** Diagram of the steps carried out in the present work to validate the qPCR experiment as a method for bacterial quantification.

To further confirm the identity and insertion direction of the sequences, the inserted fragments in the pGEM-T were sequenced using M13 forward primer (STABVIDA). Positive plasmids containing BL8A and BL18A markers were named as pEA1 and pEA3.

Each plasmid DNA was quantified using Qubit dsDNA HS Assay (Invitrogen) and plasmid copy number was determined according Gómez-Doñate et al., (2012) formula. For this purpose, molecular mass of plasmids containing each DNA marker was calculated using the Sequence Manipulation Suite (SMS) – DNA Molecular Weight (Stothard, 2000).

Gómez-Doñate et al. (2012) formula:

$$\boldsymbol{number\ of\ molecules\ of\ pGEM\text{-}T\ Easy :: insert/\mu L} = \\ = \frac{concentration\ of\ pGEM\text{-}T\ Easy :: insert\ (ng/\mu L)}{molecular\ mass\ (ng/mol)} \times 6.022 \times 10^{23}\ molecules/mol$$

Ten-fold serial dilutions (1 to $10^{-7}$) of pEA1 and pEA3 plasmids in DEPC-treated water (Sigma-Aldrich, Switzerland) were used to generate standard curves for qPCR by plotting quantification cycles (Cqs) vs. number of plasmid copies.

To test the ability of screening both markers in DNA samples, as well as to determine the LOQ, ten-fold serial dilutions (1 to $10^{-8}$) in DEPC-treated water (Sigma-Aldrich) of *B. licheniformis* 9945A and FI1 DNAs were made. Initial number of genomes in these samples was determined using the copy number calculator for realtime PCR (SciencePrimer).

Bacterial cultures in LB medium (AppliChem) of *B. licheniformis* 9945A and FI1 were also submitted to ten-fold serial dilutions (1 to $10^{-8}$), which were plated onto NA (Liofilchem) to allow CFU enumeration. DNA from each dilution was extracted using the EZNA Bacterial DNA Purification Kit (Omega Bio-Tek), following manufacturer's instructions, and used to determine the efficiency of extraction and also the LOQ value.

In order to determine the efficiency of the extraction method described in section 2.7., sample processing controls (SPCs) consisting in DNA extracted from faecal samples without (SPC0) or with bacterial pellets of known *B. licheniformis* concentration of 5, 4.52 × $10^3$ or 4.52 × $10^6$ CFUs/mg, SPC1, SPC$10^3$ or SPC$10^6$, respectively. This approach also allowed the determination of the LOQ value for faecal samples.

Each qPCR reaction of 20 µL consisted in 1 x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 0.35 µM of each primer (STAB Vida) and 1µL of each sample, which were tested in triplicate. To elute the possible presence of PCR inhibitors, which can be co-extracted with DNA from faecal samples, each faecal DNA was submitted to ten-fold dilutions (1 to $10^{-2}$) and each dilution was also tested in triplicates.

The controls included were the (i) non-template control (NTC); (ii) 9.8 ng/µL of *B. licheniformis* 9945A as positive control; (iii) an internal positive control (IPC) consisting in the sample 6 containing 9.8 ng/µL of *B. licheniformis* 9945A (IPC-6); and (iv) an control to screen for unspecific amplification consisting in the sample 6 containing 9.8 ng/µL of *B. sonorensis* LMG_21636 (Bsono-6).

The qPCR assay was carried out using CFX Connect™ Real-Time Detection System (Bio-Rad) and qPCR program consisted of 2 min at 98 ºC for polymerase activation and DNA denaturation, 40 cycles of 5 s at 98 ºC for denaturation and 30 s at 60ºC for annealing, followed of melting curve analysis starting from 65 ºC to 95 ºC, using a 0.5 ºC increment for 5 s per step.

Data were analyzed using the CFX Manager™ Software v3.1 (Bio-Rad) and the Cq determination mode used was regression. Taking into account that each marker is present as single copy per genome of *B. licheniformis*, absolute bacterial quantification in faecal samples was obtained by comparison with the standard curve and expressed as number of chromosomes per milligram of faeces.

# 3. Results

## 3.1. Identification of bacterial isolates through 16S rRNA gene sequencing

The twenty seven bacterial isolates, obtained from fish gut, were identified by 16S rRNA gene sequencing. The obtained sequences were analyzed using the RDP database, which is able to determine their similarity values in comparison to other sequences present in the database. The majority of the isolates obtained from the gut of farmed fish were presumptively identified as *B. licheniformis* (FI1, FI2, FI3, FI11, FI34, FI35, FI40, FI42, FI44, FI47, FI94, FI132, FI136, FI139, FI141, FI159, FI161, FI242 and FI268). However, some isolates identified as *B. licheniformis* also showed high similarity with *Bacillus aerius* (FI105, FI120 and FI144) or with *Bacillus oleronius* (FI152) (Table 3). Concerning the isolates FI38, FI39, FI46 and FI157, it was not possible to obtain a robust similarity to any of the *Bacillus* species reported at RDP and therefore these isolates were classified as *Bacillus* sp. (Table 3; Attachment I).

The 16S rRNA gene sequences from FI1, FI2, FI3, FI11, FI34, FI35, FI40, FI94, FI105, FI120 FI132, FI136, FI139, FI141, FI144, FI161, FI242 and FI268 were deposited in GenBank under accession numbers from KM598336 to KM598353. The 16S rRNA gene sequences from FI42, FI44, FI47, FI152 and FI159 are available in Attachment II.

**Table 3 –** Presumptive identification of spore-forming bacterial isolates obtained from farmed fish digesta samples and based on 16S rRNA gene sequencing.

| Isolate | Ribosomal Database Project (RDP) | |
|---|---|---|
| | **Presumptive Identification** | **Similarity score** |
| **FI1** | *B. licheniformis* | 1.000 |
| **FI2** | *B. licheniformis* | 1.000 |
| **FI3** | *B. licheniformis* | 1.000 |
| **FI11** | *B. licheniformis* | 1.000 |
| **FI34** | *B. licheniformis* | 0.988 |
| **FI35** | *B. licheniformis* | 1.000 |
| **FI38** | *Bacillus* sp. | 0.997 |
| **FI39** | *Bacillus* sp. | 0.997 |
| **FI40** | *B. licheniformis* | 0.991 |

**Table 3** (cont.) **–** Presumptive identification of spore-forming bacterial isolates obtained from farmed fish digesta samples and based on 16S rRNA gene sequencing.

| Isolate | RDP | |
|---|---|---|
| | **Presumptive Identification** | **Similarity score** |
| **FI42** | *B. licheniformis* | 0.973 |
| **FI44** | *B. licheniformis* | 0.925 |
| **FI46** | *Bacillus* sp. | 0.908 |
| **FI47** | *B. licheniformis* | 0.996 |
| **FI94** | *B. licheniformis* | 1.000 |
| **FI105** | *B. licheniformis*; *B. aerius* | 1.000 |
| **FI120** | *B. licheniformis*; *B. aerius* | 1.000 |
| **FI132** | *B. licheniformis* | 1.000 |
| **FI136** | *B. licheniformis* | 1.000 |
| **FI139** | *B. licheniformis* | 1.000 |
| **FI141** | *B. licheniformis* | 1.000 |
| **FI144** | *B. licheniformis*; *B. aerius* | 1.000 |
| **FI152** | *B. licheniformis*; *B. oleronius* | 1.000 |
| **FI157** | *Bacillus* sp. | 0.995 |
| **FI159** | *B. licheniformis* | 0.996 |
| **FI161** | *B. licheniformis* | 1.000 |
| **FI242** | *B. licheniformis* | 1.000 |
| **FI268** | *B. licheniformis* | 1.000 |

### 3.2. Taxa-specific regions and DNA markers

Following Flowchart 1 (Fig. 4), the overlap of CUPID and Insignia's outputs obtained using a C+.program allowed to retrieve 11 putatively specific ORFs for *B. licheniformis* ATCC_14580. A follow-up BLAST analysis of these regions revealed that 5 ORFs were specific for this species, however, the design of specific primers presenting the maximum quality score was only possible for one region – marker BL5B (Table 4), which is specific to *B. licheniformis* but is not present in all sequenced strains.

Specific regions for *B. licheniformis* DSM_13 were calculated using only Insignia (Flowchart 2; Fig. 4). In order to account only for annotated coding regions, the outputted regions were overlapped with the complete list of proteins from this organism (NCBI database) and 110 putative specific ORFs for *B. licheniformis* were selected. From these, only 27 were confirmed as specific by the BLAST analyses and the design of specific primers presenting the maximum quality score was possible for 3 different

regions – markers BL8A, BL13C and BL18A (Table 4), which are specific and transversal to all *B. licheniformis* strains completely sequenced.

ORFs localization, primers and amplicon size of each selected marker are summarized in Table 4.

**Table 4 –** *B. licheniformis* specific markers, the corresponding ORFs and primers used with the expected amplicon size.

| ORF | Marker | [a]Primers | Primers sequence (5' – 3') | Amplicon size (bp) |
|---|---|---|---|---|
| BL00303 | BL5B | Fwd | CGCTCACCATATGCACAGCTCT | 332 |
| | | Rev | CGGTTTATCGCTTGAGACTCGG | |
| *serA2* | BL8A | Fwd | TCACAACCCGTTGACGACAA | 247 |
| | | Rev | CGTGTCCGAGTGTGCGTTATAT | |
| BLi00806 | BL13C | Fwd | TTGTGCGTATCTCCGGGCCA | 376 |
| | | Rev | AGGCATTGTCCCGATGGTGG | |
| *ligD* | BL18A | Fwd | GTCAACGACACAATTTCCCCGT | 216 |
| | | Rev | AGCTCCCTCAGGCGGCAATT | |

[a] Fwd – primer forward; Rev – primer reverse.

### 3.3. *In silico* analyses of DNA markers

Comparative genomics analyses were carried out in order to disclose the genomic stability of the regions where the selected markers are located, using the chromosome of *B. licheniformis* type strain ATCC_14580 as reference. Based on the location of the markers in relation to several features annotated in the chromosome and generally associated with genomic mobility, such as phage related ORFs, IS, recombinases, integrases, transposases and tRNAs, the analyses showed that the DNA markers were within conserved chromosomal regions (Fig. 6).

Furthermore, the GC content for most of the coding regions where markers are located is approximate to the overall chromosomal GC content (46.2 %) (Fig. 6; Table 5). To further sustain the genomic stability of the chosen markers, the normalized Codon Adaptation Index ratio (CAI/eCAI) for each region was higher than the value 1 and similar to the CAI/eCAI values calculated for *B. licheniformis* HKs used for MLST typing (Table 5). Altogether these data support the hypothesis that the selected DNA markers are included in stable genomic regions, therefore suitable to be used as identification markers for *B. licheniformis* species.

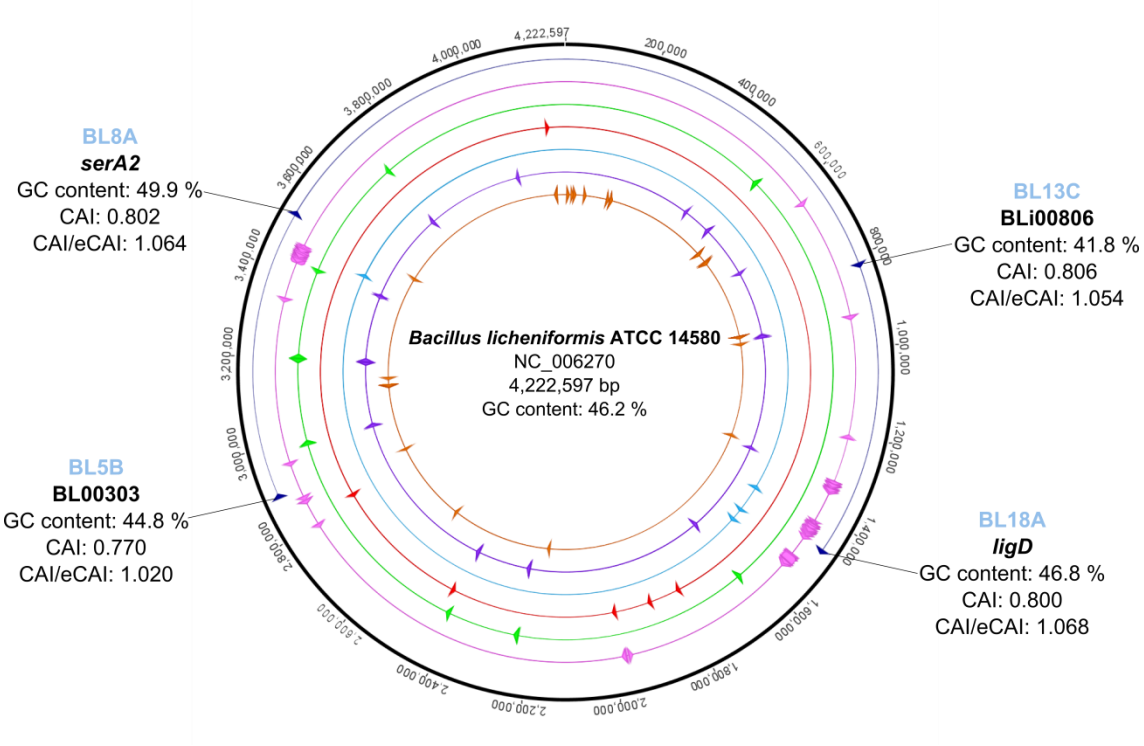



**Fig. 6 –** Genome map of *B. licheniformis* ATCC_14580 pinpointed with DNA markers (dark blue), phage related ORFs (pink), IS (green), recombinases (red), integrases (blue), transposases (purple) and tRNAs (brown). The locus tag, GC content, CAI and normalized CAI (CAI/eCAI) values are shown for each coding region containing the DNA markers.

**Table 5 –** The values of GC content, CAI, eCAI and CAI/eCAI ratio obtained for each ORF containing the different DNA markers and for seven HKs commonly used for *B. licheniformis* MLST typing.

| Locus tag | Marker | % GC | CAI | eCAI | CAI/eCAI |
|---|---|---|---|---|---|
| BL00303 | BL5B | 44.8 | 0.770 | 0.755 | 1.020 |
| *serA2* | BL8A | 49.9 | 0.802 | 0.754 | 1.064 |
| BLi00806 | BL13C | 41.8 | 0.806 | 0.765 | 1.054 |
| *ligD* | BL18A | 46.8 | 0.800 | 0.749 | 1.068 |
| *Adk* | HK | 44.6 | 0.779 | 0.750 | 1.039 |
| *ccpA* | HK | 47.8 | 0.790 | 0.746 | 1.059 |
| *recF* | HK | 42.8 | 0.794 | 0.766 | 1.037 |
| *rpoB* | HK | 48.6 | 0.771 | 0.744 | 1.036 |
| *spo0A* | HK | 48.9 | 0.799 | 0.740 | 1.080 |
| *sucC* | HK | 48.4 | 0.822 | 0.745 | 1.103 |

Whole genome syntenic plots were constructed to compare the genome of *B. licheniformis* ATCC_14580 with *B. licheniformis* DSM_13 and with *B. licheniformis* 9945A (Fig. 7).

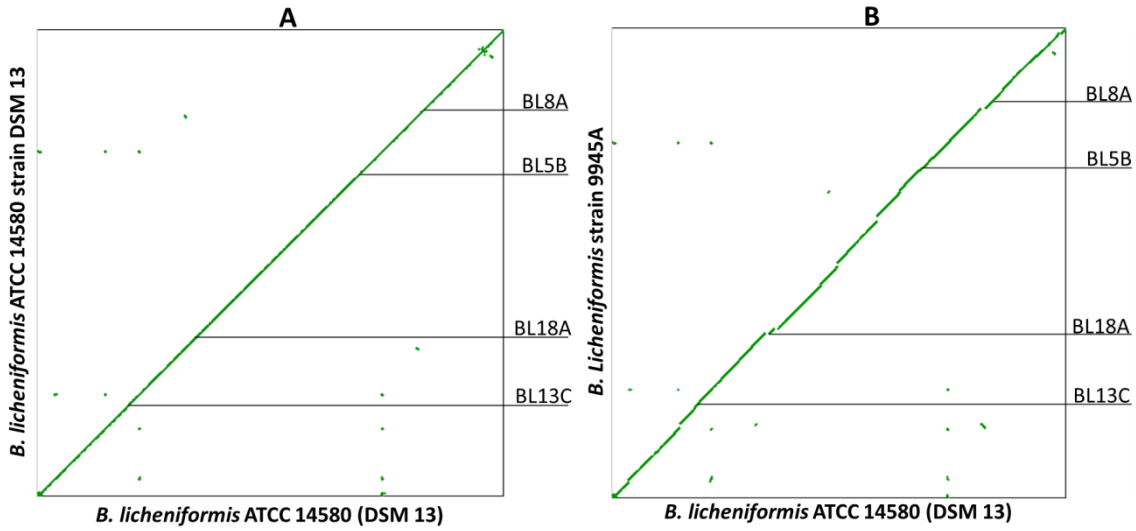



**Fig. 7 –** Synteny maps, obtained using the SynMap tool from CoGe, showing the approximate location of the selected DNA markers. Comparison of *B. licheniformis* ATCC_14580 with *B. licheniformis* DSM_13 (A) and with *B. licheniformis* 9945A (B).

Detailed synteny maps confirmed that ORFs *serA2*, BLi00806 and *ligD* containing BL8A, BL13C and BL18A markers, respectively, are located inside syntenic regions (Attachment III), which supports the hypothesis that the selected DNA markers are located in conserved and stable chromosomal regions. More importantly, the synteny profile of markers BL8A, BL13C and BL18A, increases the likelihood of these markers being common to all strains of *B. licheniformis* and therefore good species-specific markers, i.e. useful to identify any *B. licheniformis* lineage, regardless the species infrasubspecific diversity. The synteny analysis of the ORF BL00303, containing BL5B marker, has shown that this marker is conserved between *B. licheniformis* ATCC_14580 and *B. licheniformis* DSM_13, but not between *B. licheniformis* ATCC_14580 and *B. licheniformis* 9945A, since this ORF is not present in strain 9945A (Attachment IV), which means that contrary to the other markers, BL5B marker is not transversal to all representatives of *B. licheniformis*.

### 3.4. Experimental validation of *B. licheniformis* species-specific DNA markers

Validation by PCR confirmed the specificity of the four DNA markers towards *B. licheniformis* species (Fig. 8). Amplification was obtained, when using markers BL8A, BL13C and BL18A, with DNA from all tested *B. licheniformis* strains (9945A and isolates FI1, FI2, FI3 and FI11). On the contrary, there was no specific amplification

with DNA of closely related species, namely *B. sonorensis* LMG_21636, *B. subtilis* 168, *B. amyloliquefaciens* LMG_9814, *B. atrophaeus* LMG_16797, *B. pumilus* ATCC_7061 and *B. megaterium* ATCC_19213. The same was observed when using the BL5B marker, that with the exception for *B. licheniformis* 9945A, in which this marker was not amplified, the results showed specific amplification of the other four strains of *B. licheniformis*, namely FI1, FI2, FI3 and FI11, and unspecific amplification of the other non-*B. licheniformis* species assayed as negative controls (Fig. 8).

The identity of the amplicons obtained with isolate FI1 for all markers was confirmed by sequencing (Attachment V).

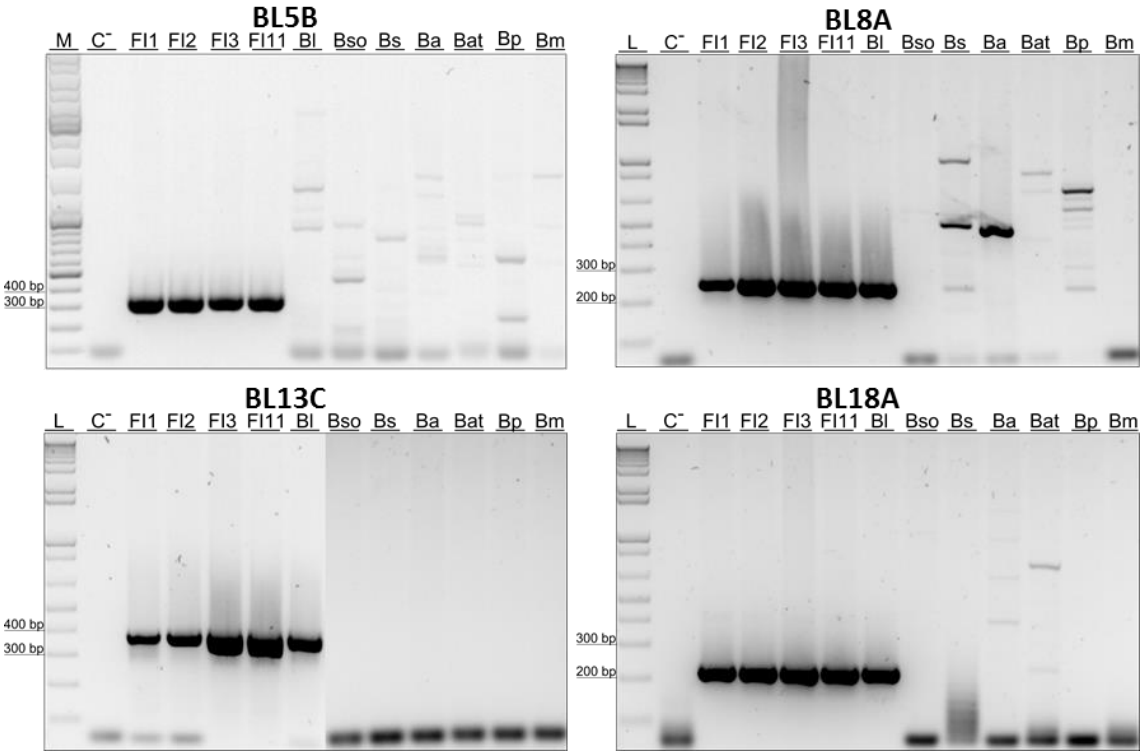



**Fig. 8 –** PCR validation of the DNA markers (BL5B, BL8A, BL13C and BL18A) using *B. licheniformis* isolates (FI1, FI2, FI3 and FI11) and strain 9945A (BI) as positive controls. *B. sonorensis* LMG_21636 (Bso), *B. subtilis* 168 (Bs), *B. amyloliquefaciens* LMG_9814 (Ba), *B. atrophaeus* LMG_16797 (Bat), *B. pumilus* ATCC_7061 (Bp) and *B. megaterium* ATCC_9213 (Bm) were used as negative controls. $C^-$ – negative control of PCR reaction; M – GeneRuler™ DNA Ladder Mix (Thermo Scientific); L – 1 Kb Plus DNA Ladder (Life Technologies).

### 3.5. Detection of *B. licheniformis* in faecal samples

In order to validate the selected markers for direct detection of *B. licheniformis* in environmental samples, i.e. without enrichment in culture, total DNA was extracted from faecal samples of gilthead seabream fed with diets differently enriched with FOS.

Using the BL5B marker, positive amplification was achieved with samples 1, 4, 6, 10 and 12, while the BL8A marker was present in all of them. Samples 3, 9 and 12 were positive for BL13C marker and the BL18A marker was observed in almost all samples, with exception for 5, 7 and 8 (Fig. 9). Regardless the fact that not all the four markers were amplified in all 12 faecal samples tested, these results suggested that *B. licheniformis* was present in all the faecal samples, whatever the diet administered to gilthead seabream.

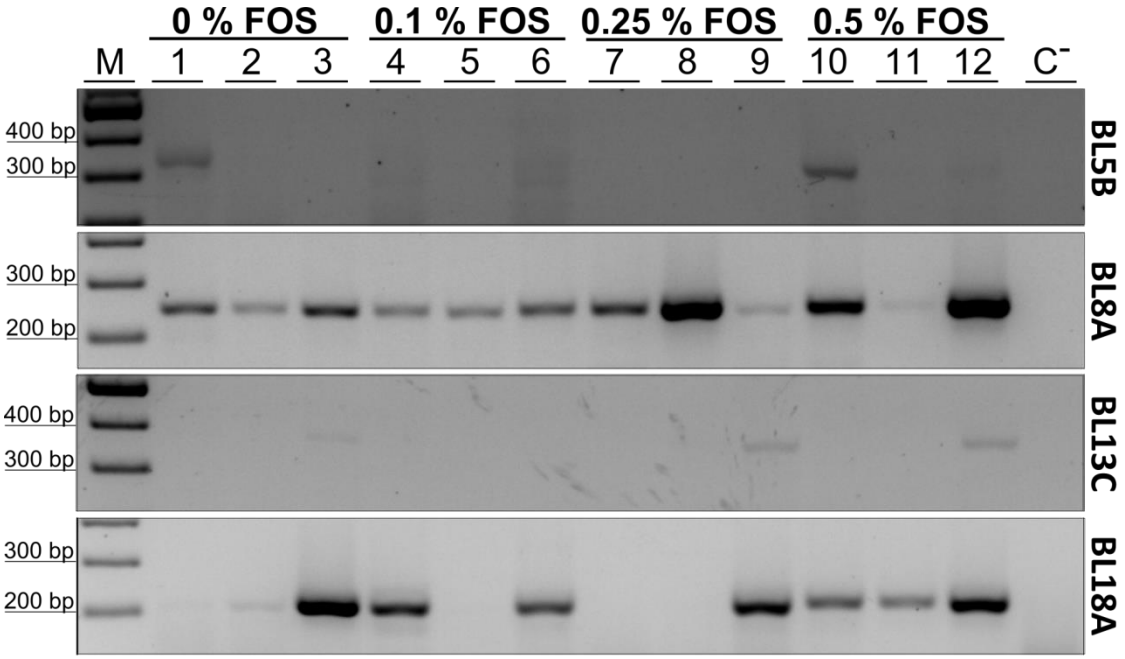



**Fig. 9 –** PCR screen of the different selected markers (BL5B, BL8A, BL13C and BL18A) in DNA extracted from faecal samples obtained from gilthead seabream fed with diets differently enriched with FOS. $C^-$ – negative control of PCR reaction. M – GeneRuler™ DNA Ladder Mix (Thermo Scientific).

### 3.6. Identification of *B. licheniformis* isolates through Multiplex PCR

To increase the consistency of PCR identification, a multiplex PCR was optimized using four *B. licheniformis* isolates (FI1, FI2, FI3 and FI11) and *B. licheniformis* 9945A. Having into consideration the amplicon size, from 216 to 376 bp, and the narrow difference between them, as low as 31 bp, to separate accurately the PCR amplicons, 8 % polyacrylamide gels were used. The results showed that all the four *B. licheniformis* specific-markers (BL5B, BL8A, BL13C and BL18A) were successfully amplified using DNA from the four isolates and no unspecific amplification was detected. As expected, the BL5B marker was not amplified with DNA from *B. licheniformis* 9945A, contrary to the other three markers (Fig. 10).

The multiplex PCR was further used to assess the identity of putative *B. licheniformis* isolates (FI34, FI35, FI38, FI39, FI40, FI42, FI44, FI46 and FI47), which were preliminary identified as *Bacillus* sp. by 16S rRNA gene sequencing. The results showed that six isolates (FI34, FI35, FI40, FI42, FI44 and FI47) were positive for all markers, suggesting that they belong to *B. licheniformis* species. On the contrary, none of the markers was amplified in isolates FI38, FI39 and FI46, indicating that these bacilli are not *B. licheniformis* (Fig. 11).

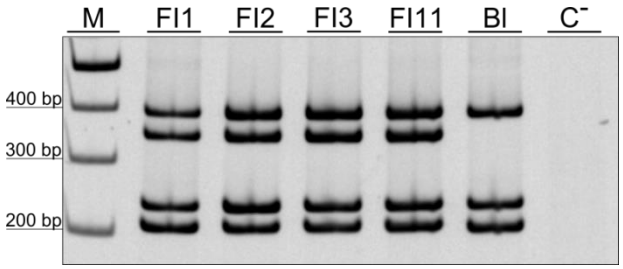



**Fig. 10 –** Testing for a multiplex PCR targeting the four markers specific for *B. licheniformis*, using *B. licheniformis* isolates (FI1, FI2, FI3 and FI11) and *B. licheniformis* 9945A (BI) as templates. $C^-$ – negative control of PCR reaction. M – GeneRuler™ DNA Ladder Mix (Thermo Scientific).

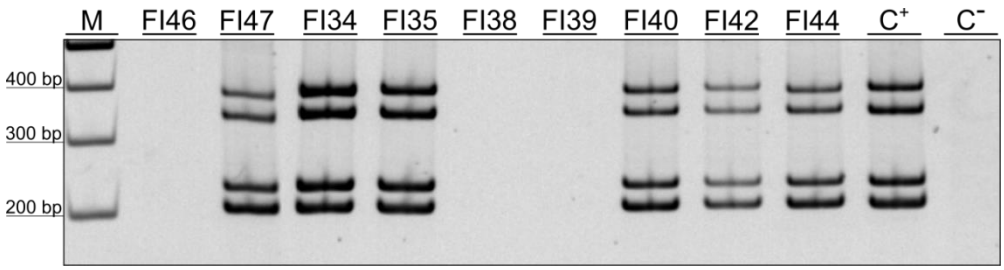



**Fig. 11 –** Multiplex PCR to screen the presence of the specific markers for *B. licheniformis* in fish isolates. $C^+$ – positive control (FI1); $C^-$ – negative control of PCR reaction. M – GeneRuler™ DNA Ladder Mix (Thermo Scientific).

Interestingly, *B. licheniformis* strains are frequently characterized by a lichen-like colony morphology (Fig. 12). This macroscopic trait allowed a preliminary identification of putative *B. licheniformis* isolates, obtained from faecal samples of European seabass. This approach allowed to identify 14 isolates (FI94, FI105, FI120, FI132, FI136, FI139, FI141, FI144, FI152, FI157, FI159, FI242, FI268 and FI282) as putative *B. licheniformis*, which were assayed for the presence of *B. licheniformis* specific markers. Multiplex PCR results showed that the four markers (BL5B, BL8A, BL13C and BL18A) were amplified in eight out of the 14 selected isolates (FI132, FI136, FI139, FI141, FI152, FI159, FI242 and FI268), strongly suggesting that these isolates were indeed *B. licheniformis*. For four other isolates (FI94, FI105, FI120 and FI144), only marker BL5B was unamplified. Having in mind that this marker was previously shown

not to be present across all *B. licheniformis* including the strain 9945A, these results still indicate that these four isolates were *B. licheniformis*. Finally, no amplification was obtained for isolates FI157 and FI282 whatever the marker, suggesting that these two isolates are not *B. licheniformis* (Fig. 13).

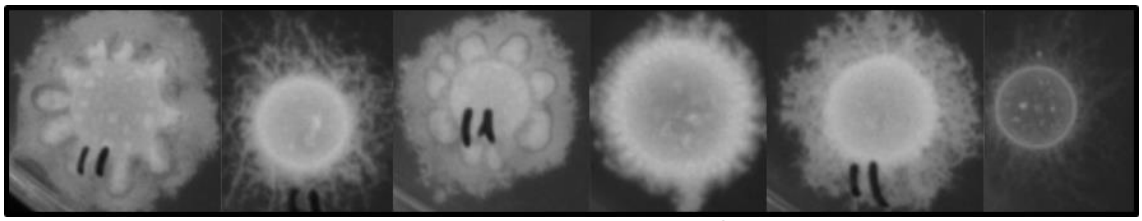


**Fig. 12 –** Distinct morphology of *B. licheniformis* colonies (Image credits: Cláudia Serra).

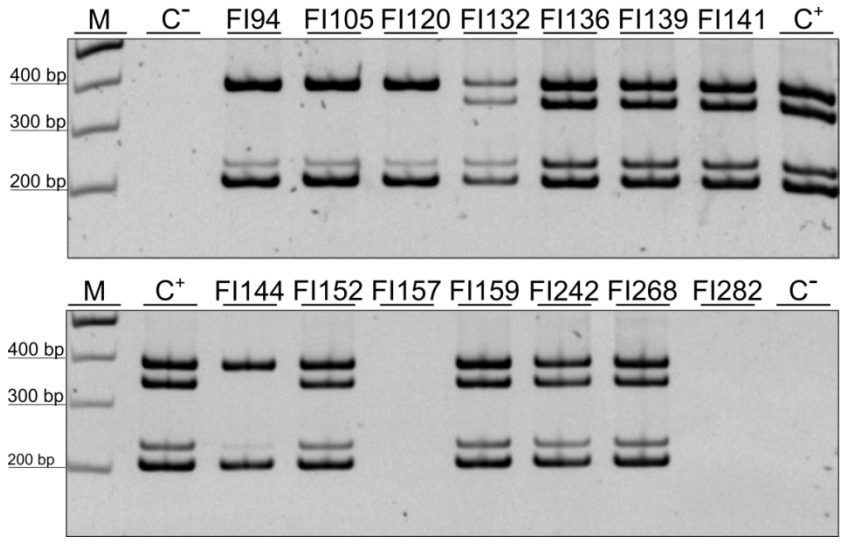



**Fig. 13 –** Multiplex PCR targeting specific markers for *B. licheniformis* in putative *B. licheniformis* distinct colonies. $C^+$ – positive control (FI1); $C^-$ – negative control of PCR reaction. M – GeneRuler$^{TM}$ DNA Ladder Mix (Thermo Scientific).

### 3.7. *B. licheniformis* quantification using qPCR

Aiming to implement a qPCR procedure to quantify *B. licheniformis* in faecal samples of farmed fish, markers BL8A and BL18A were preferably chosen as the reference markers for qPCR. This choice had into account that from the four *B. licheniformis* specific markers (BL5B, BL8A, BL13C and BL18A) validated for identification, BL8A and BL18A were the markers with a more convenient size for qPCR, 247 and 216 bp respectively, and were also the markers that performed better for PCR of environmental faecal samples from gilthead seabream (Fig. 9).

In order to make a calibration curve for qPCR that would allow the quantification of these markers in environmental samples, BL8A and BL18A markers were inserted into pGEM-T originating plasmids pEA1 containing the BL8A marker and pEA3 containing BL18A marker. The insertion of both markers in pEA1 and pEA3 was confirmed by restriction digestion and sequencing (Fig. 14).

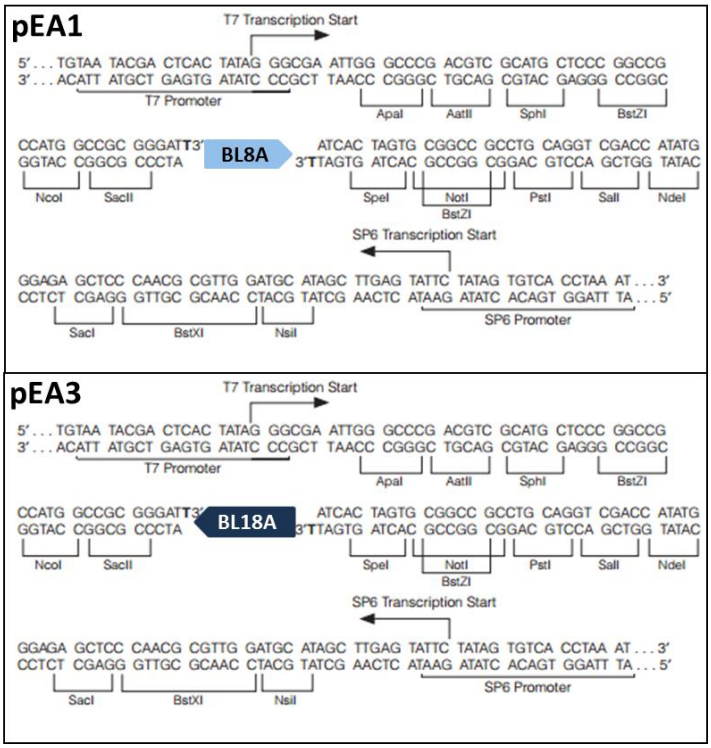



**Fig. 14 –** Multiple cloning site sequence of pGEM-T where amplicons of BL8A and BL18A were inserted, originating pEA1 and pEA3 respectively. BL8A in the forward direction and BL18A in the reverse direction.

Both plasmids pEA1 and pEA3 hosting, respectively, markers BL8A and BL18A, were cloned in *Escherichia coli* DH5α. Plasmid DNA was then extracted and quantified using the Qubit dsDNA HS Assay to determine the plasmid copy number per µL for both plasmids using a formula from Gómez-Doñate et al. (2012): pEA1 = $1.36 \times 10^{10}$ molecules/µL and pEA3 = $1.55 \times 10^{10}$ molecules/µL. Ten-fold dilutions of these plasmid samples were prepared for use in qPCR, aiming to build reliable standard curves for each marker, that would allow the quantification of both markers BL8A and BL18A in environmental DNA samples. The obtained standard curves revealed an efficiency of 106.2 % and a coefficient of determination ($R^2$) of 0.990 when using pEA1, and an efficiency of 95.3 % and an $R^2$ of 0.999 when using pEA3, which is in conformity with the requirements needed for qPCR calibration curve (Fig. 15).

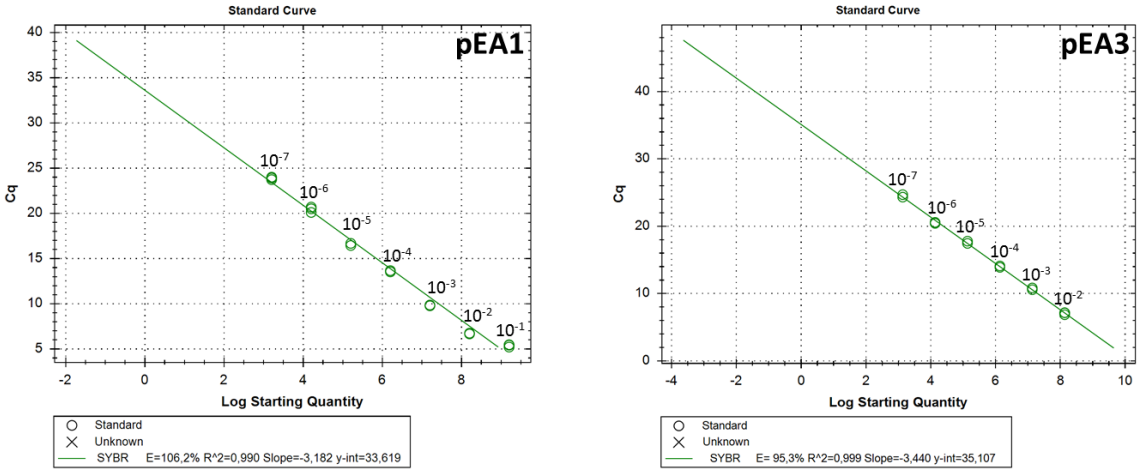



**Fig. 15 –** Standard curves obtained for BL8A (pEA1) and BL18A (pEA3) markers. Dilutions used to construct the standard curves and corresponding efficiencies (E) and $R^2$ are represented.

To determine the potential of qPCR to amplify the markers in DNA samples and determine the LOQ, ten-fold serial dilutions of DNA from *B. licheniformis* FI1 and of strain 9945A were used. This limit was determined as the highest dilution for which a consistent Cq value was obtained. Having into account that both markers occur as single copies in *B. licheniformis* chromosome, the bacterial LOQ for the BL8A marker was determined as 1 chromosomes/µL for strain 9945A and 70 chromosomes/µL for isolate FI1. For BL18A marker, the bacterial detection limit was 70 chromosomes/µL for both strains (Table 6). Consequently, the LOQ value for DNA dilutions was set between 1 and 70 chromosomes/µL.

Additionally, to assess the efficiency of the bacterial DNA extraction procedure and to determine the LOQ when using *B. licheniformis* cells instead of chromosomal DNA, ten-fold serial dilutions of *B. licheniformis* 9945A and FI1 liquid cultures were used to extract DNA and simultaneously to count the number of cells by CFU. The CFU assays allow to calculate the initial copy number of the undiluted cultures as $2 \times 10^6$ and $8.2 \times 10^5$ CFUs/µL for *B. licheniformis* 9945A and FI1, respectively. Using this approach, the LOQ value was estimated at 2 CFUs/µL for strain 9945A for both markers, while for FI1 isolate the LOQ value estimated was 8 CFUs/µL and 1 CFUs/µL for BL8A and BL18A markers, respectively (Table 7). Accepting as premises that there is a single chromosome per *B. licheniformis* cell and that all *B. licheniformis* cells in culture were viable, these results allowed to set the LOQ for *B. licheniformis* between 1 and 8 chromosomes/µL.

**Table 6 –** qPCR bacterial LOQ for markers BL8A and BL18A, using serial dilutions of *B. licheniformis* genomic DNA.

| Sample (DNA serial dilutions) | BL8A | | BL18A | |
|---|---|---|---|---|
| | **E (%) / $R^2$** | **LOQ** [a](chr./µL) | **E (%) / $R^2$** | **LOQ** [a](chr./µL) |
| *B. licheniformis* 9945A | 96.0 % / 0.998 | 1 | 95.5 % / 0.998 | 70 |
| FI1 | 66.2 % / 0.990 | 70 | 69.5 % / 0.982 | 70 |

[a] chr. – chromosomes

**Table 7 –** qPCR bacterial LOQ for markers BL8A and BL18A, using serial dilutions *B. licheniformis* cultures.

| Sample (Culture serial dilutions) | BL8A | | BL18A | |
|---|---|---|---|---|
| | **E (%) / $R^2$** | **LOQ** (CFUs/µL) | **E (%) / $R^2$** | **LOQ** (CFUs/µL) |
| *B. licheniformis* 9945A | 89.0 % / 0.969 | 2 | 93.5 % / 0.975 | 2 |
| FI1 | 74.6 % / 0.901 | 8 | 85.1 % / 0.872 | 1 |

To further validate this culture-independent quantification procedure of *B. licheniformis*, and to investigate the possible inhibitory effect of molecules co-extracted during DNA extraction from faecal samples, qPCR was carried out using as template DNA extracted from faecal samples of gilthead seabream fed with diets differently enriched in FOS. The qPCR standard curves testing faecal samples presented efficiencies varying from 91.7 % to 100.2 % and a coefficient of determination ($R^2$) ranging from 0.966 to 0.985 for the BL8A marker. Concerning BL18A, the efficiencies varied between 98.9 % and 104.9 % and the $R^2$ from 0.958 to 0.989. By comparing the fluorescence measured by qPCR for each faecal sample with these standard curves, it was possible to infer the number of *B. licheniformis* chromosomes per mg of faecal sample.

qPCR data were analyzed for BL8A and BL18A independently and the number of marker copies per mg ± SD was calculated for each sample taking into account the results of a single sample dilution. Thus, to choose the dilution for which this value was calculated, two criteria were followed, firstly select the higher dilution for which the Cq value can be assessed, and secondly choose the dilution which showed the minimum variations between triplicates. Bacterial quantification could be inferred from marker copy number determined by qPCR, since each marker is present as single copy per chromosome of *B. licheniformis*.

To determine the efficiency of the faecal DNA extraction method, faecal samples were spiked with known amounts of *B. licheniformis* cells, namely 0, 5, $4.52 \times 10^3$ and $4.52 \times 10^6$ CFUs/mg, corresponding to samples SPC0, SPC1, SPC$10^3$ and SPC$10^6$, respectively. Quantification by qPCR was performed through comparison to the standard curves determined for each assay (E= 91.7 % and $R^2$= 0.973 for marker BL8A; E= 98.9 % and $R^2$= 0.961 for maker BL18A). The results showed that only the faecal samples spiked with *B. licheniformis* corresponding to $4.52 \times 10^6$ CFUs/mg, i.e. sample SPC$10^6$, was close to the value of quantification determined by qPCR and corresponding to $8.715 \times 10^5$ CFUs/mg for marker BL8A and $8.173 \times 10^5$ CFUs/mg for marker BL18A (Fig. 16). These suggest that the cut-off limit for quantification of *B. licheniformis* in faecal samples above $10^6$ CFUs/mg.

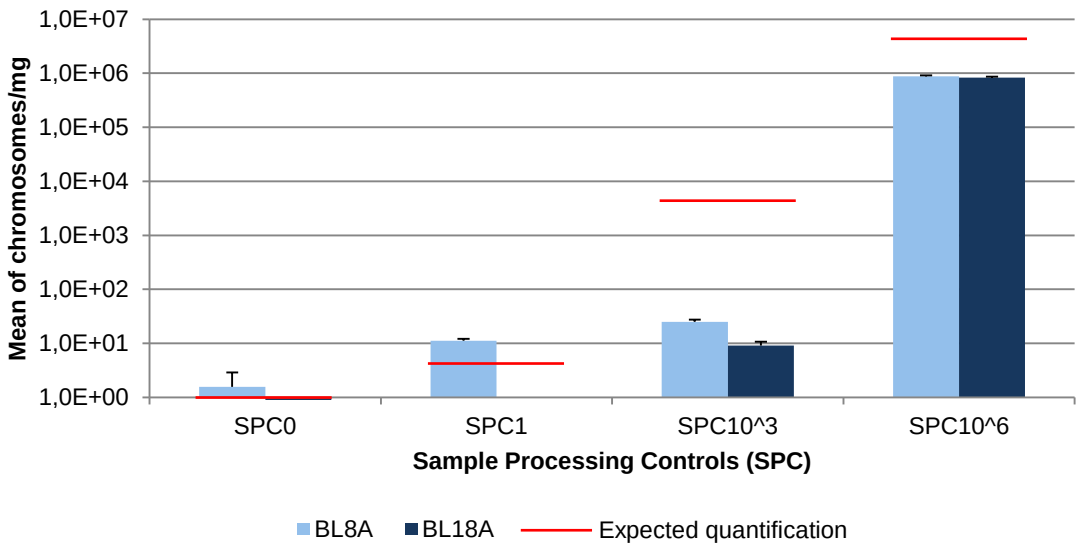



**Fig. 16 –** qPCR quantification of *B. licheniformis* in spiked faecal samples, designated as SPCs, obtained using markers BL8A and BL18A. SPCs consisted in DNA from faecal samples co-extracted with known concentrations of bacterial cultures: 0 (SPC0), 1 (SPC1), $10^3$ (SPC10^3) or $10^6$ (SPC10^6) CFUs/mg. The real CFU quantification is highlight in red as CFUs/mg. qPCR results are expressed in average of *B. licheniformis* chromosomes/mg ± SD.

In order to investigate the presence of qPCR inhibitors in faecal samples, a known amount of *B. licheniformis* 9945A chromosomal DNA (9.8 ng chromosomal DNA/µL), determined by Qubit dsDNA Assay and estimated as $2.1 \times 10^6$ chromosomes/µL, was added to sample 6 (i.e. faecal sample corresponding to gilthead seabream fed with 0.1 % FOS). Quantification using qPCR was obtained by comparing the qPCR data with the standard curves obtained in the same qPCR reaction and presenting an efficiency of 91.7 % and a $R^2$ of 0.973 for marker BL8A, and an efficiency of 98.9 % and a $R^2$ of

0.961 for marker BL18A. The results allowed to estimate a concentration of *B. licheniformis* as 4.7 × $10^6$ chromosomes/µL for marker BL8A and 3.6 × $10^6$ chromosomes/µL for marker BL18A (Fig. 17). These results showed that the qPCR quantification of *B. licheniformis* were within the same order of magnitude as the number of *B. licheniformis* chromosomes determined by DNA quantification using Qubit dsDNA Assay, i.e. $10^6$ chromosomes/µL, which suggests the absence of qPCR inhibitors in gilthead seabream faecal samples.

As negative control, the same sample 6 (corresponding to gilthead seabream fed with 0.1 % FOS) was also spiked with 9.8 ng chromosomal DNA/µL (estimated as 1.9 × $10^6$ chromosomes/µL) of *B. sonorensis* LMG_21636, and designated as sample Bsono-6. No additional amplification was recorded with this sample, further confirming the specificity of the qPCR procedure to quantify *B. licheniformis* (Fig. 17).

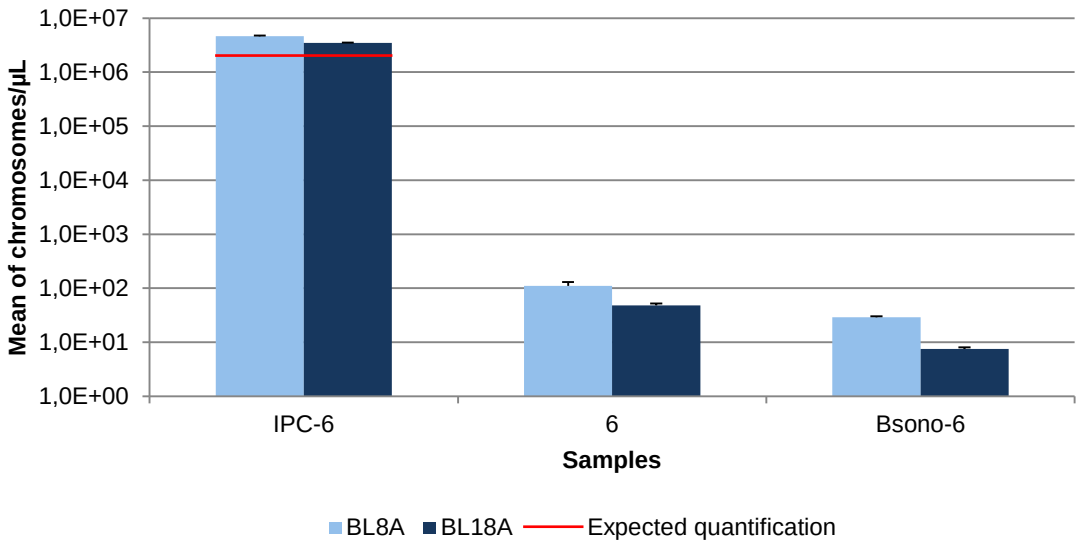



**Fig. 17 –** qPCR quantification of *B. licheniformis* in gilthead seabream faecal sample (Sample 6) using markers BL8A and BL18A to assess the presence of qPCR inhibitors (IPC-6) and the reaction specificity (Bsono-6). Minimum expected quantification for IPC-6 is highlighted in red. Results are expressed in average of *B. licheniformis* chromosomes/µL ± SD.

To disclose a possible change in *B. licheniformis* gilthead seabream gut population triggered by diets supplemented with different concentrations of FOS, DNA extracted from faecal samples of these fish fed with diets differently enriched with FOS (0, 0.1 %, 0.25 % and 0.5 % FOS) was used as template for qPCR. The standard curves of these experiments had an efficiency of 91.9 % and 100.2 %, and a corresponding $R^2$ of 0.966 and 0.985 for BL8A marker, while for marker BL18A, the efficiency obtained was 104.9

% and 99.3 % and a corresponding $R^2$ of 0.958 and 0.989. The qPCR results obtained for each of the samples were below the quantification threshold of qPCR determined with the SPCs (Fig. 18), which suggested that none of the diets assayed was able to uphold the growth of gut resident *B. licheniformis* for measurable values, i.e. above the quantification threshold. Regardless these data, one cannot exclude changes in the gut resident *B. licheniformis* population due to the different diets, although these changes would have to be lower that the quantification threshold of qPCR.

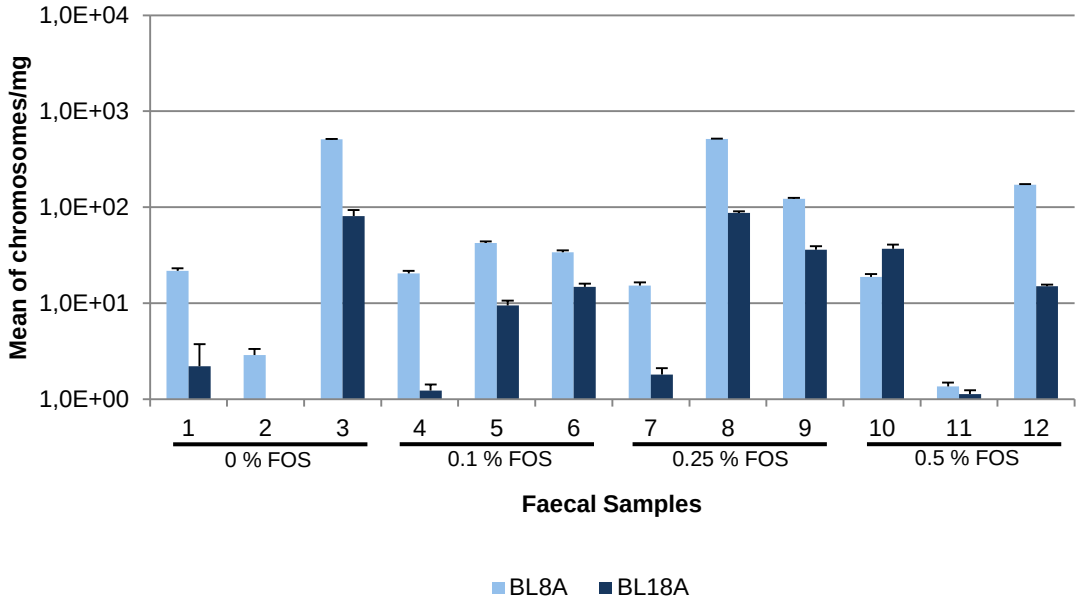



**Fig. 18 –** qPCR quantification of *B. licheniformis* in gilthead seabream faecal samples using markers BL8A and BL18A to quantify *B. licheniformis* chromosomes/µL. Results are expressed in average of *B. licheniformis* chromosomes/µL ± SD. Diets applied to the fish from which samples were collected are also pointed.

# 4. Discussion

The study of microbial communities and their interactions with biotic and abiotic elements of the environment is the focus of microbial ecology (ISME, 2013). These communities, frequently composed by numerous populations of a taxonomically broad range of bacteria with distinct features, are complex and despite the contributions made over the last years by high-resolution molecular methods, particularly metagenomics, the dynamics of microbial populations is still poorly understood (Prosser et al., 2007). The gut microbiota of humans is probably, at the moment, the microbial community more deeply studied, and at the origin of major scientific breakthroughs that change the paradigm by which we look at the importance of microorganisms in the gut. Currently is widely acknowledged that human gut microbiota is implicated in chronic and auto-immune diseases, in obesity, in controlling pathogenic bacteria and modulating gut microbiota (Clemente et al., 2012; Lozupone et al., 2012). These contributions led to the researchers interest in improving fish farming practices by start looking the still poorly characterized microbiota of farmed fish (Burr et al., 2005). In aquaculture research, with the exception for only few studies focused on the functional relationships between fish and their gut microbiota (Tapia-Paniagua et al., 2010), not much is known about the interactions between different microbiota populations, their role in metabolizing diet compounds; their inhibitory capacity over pathogens, or their ability to modulate gut microbiota. In this regard, the importance that probiotics gained to improve fish farming production has call attention to comprehend their specific roles and dynamics within the gut, i.e. beyond the causality data that has been up until now the major argument supporting the beneficial effects of probiotics in aquaculture production.

Previous studies carried out by our group (MDE) in collaboration with NUTRIMU, using different farmed fish species, revealed a predominance of *B. licheniformis* isolates in fish digesta (unpublished work), which suggested that the microbiota of these fish is particularly enriched for this species. Interestingly, *B. licheniformis* is also one of the most commonly used probiotics for aquaculture (Moriarty, 2003). These two reasons and the need to improve the tools to track microbiota relevant bacteria,

autochthonous or added as probiotics, in digesta samples aiming to understand their functional role and dynamics on the fish microbiota, led us to focus on *B. licheniformis* as a convenient model organism for the present work.

Identification of *B. licheniformis* is commonly carried out by culture-based procedures (Gordon et al., 1973) that are dependent on laborious and time-consuming techniques. So far, culture independent detection techniques for *B. licheniformis* in environmental samples have not been reported to date. A major challenge to develop molecular methods for detection of bacterial species is to identify taxa-specific loci able to discriminate the target bacteria from closely related species.

Bioinformatics tools have been developed to allow the *in silico* selection of taxa-specific markers, namely CUPID (Mazumder et al., 2005) for specific proteins and Insignia (Phillippy et al., 2009) for specific DNA regions, which can be used for identification and culture-independent monitoring of the target taxa. More recently, these utilities have been successfully used for the selection of several novel taxa-specific markers that have shown to be highly discriminatory and more importantly able to be used for the direct detection of the target microorganism in complex environmental samples (Albuquerque et al., 2012a, 2012b; Almeida et al., 2013).

To further confirm the identity of *B. licheniformis* isolates and also to be able to screen these bacteria in the gut of farmed fish, *B. licheniformis* specific DNA markers were selected using both CUPID and Insignia, according a methodology described by Albuquerque et al. (2012b), which resulted in a single marker (BL5B); or using exclusively Insignia, that allowed to retrieve dozens of putative DNA signatures. These putative *B. licheniformis* specific DNA markers were filtered to include exclusively sequences present in annotated ORFs, which are usually associated with more stable genomic regions and consequently with a higher probability of being conserved between different strains of the same species.

Since both databases are not updated in real time, in order to further validate the specificity of the selected signatures for *B. licheniformis* and to decide on the most promising DNA signatures retrieved by Insignia, a BLAST analysis was carried out. From this analysis, four *B. licheniformis* specific DNA markers were chosen to follow for experimental validation by PCR: BL5B, BL8A, BL13C and BL18A (Table 4). Two of these markers are in ORFs annotated as hypothetical proteins (BL5B and BL13C), and the other two markers, BL8A and BL18A belong, respectively, to genes coding for a D-3-phosphoglycerate dehydrogenase (*serA2*) and an ATP-dependent DNA ligase (*ligD*).

Comparative genomics analyses were also carried out to better infer the genomic stability of the loci containing the makers, according to a workflow previously optimized (Albuquerque et al., 2012a, 2012b; Almeida et al., 2013). These analyses showed that all selected markers are located within conserved genomic regions, i.e. away from elements associated with genomic mobility (Fig. 6 and 7). Furthermore, from all the *B. licheniformis* strains that made possible a detailed genomic comparison, i.e. strains with the genome fully sequenced, it was evident that, contrary to markers BL8A, BL13C and BL18A, which were shown to be specific and transversal to all completely sequenced strains of this species (Attachments III and IV), marker BL5B was specific but absent from strain 9945A, i.e. not transversal to all sequenced strains of *B. licheniformis* (Attachment IV).

Experimental validation by PCR confirmed the specificity of the selected markers, since markers BL8A, BL13C and BL18A provided amplification for the five *B. licheniformis* strains assayed and no specific amplification was observed with bacteria from closely related species, namely *B. sonorensis* LMG_21636, *B. subtilis* 168, *B. amyloliquefaciens* LMG_9814, *B. atrophaeus* LMG_16797, *B. pumilus* ATCC_7061 and *B. megaterium* ATCC_19213. The same results were obtained for BL5B marker with the exception that no amplification was obtained with strain 9945A (Fig. 8), confirming comparative genomics data.

To ascertain about the utility of these new markers for culture-independent detection of *B. licheniformis*, total DNA extracted from gilthead seabream faecal samples was used as template for PCR with the four *B. licheniformis* specific DNA markers. The results showed that, apart from the fact not all the 12 faecal samples tested were positive for all the four markers, all the samples were positive at least for one of the markers, with BL8A as the only marker amplified in all samples tested (Fig. 9). Although being difficult to determine, one might hypothesize that the different efficiency for these four markers might be related with the differential inactivation of the primers by presumably annealing to unspecific DNA, but not resulting in PCR amplification. It is widely acknowledged that extraction of DNA from environmental samples, including faecal samples, frequently result in the co-extraction of PCR inhibitors that impair amplification (Alaeddini, 2012; Pontiroli et al., 2011; Schrader et al., 2012). The fact that the PCR reactions were not inhibited for marker BL8A seems to indicate that the inhibition was at the primers and not at the PCR reaction itself. Altogether, and due to the redundancy achieved by using four markers, we conclude that all the faecal samples tested were positive for the presence of *B. licheniformis*. Having in mind that

there are no molecular and culture-independent procedures available to detect *B. licheniformis*, these markers open new opportunities to track *B. licheniformis* in environmental samples.

Besides allowing to monitor the presence of this species in faecal samples, *B. licheniformis* specific markers were also used in a multiplex PCR approach to identify rapidly and unequivocally culture isolates obtained from faecal samples. From the 23 isolates selected as probable *B. licheniformis*, 18 isolates were amplified by the implemented multiplex PCR (Fig. 11 and 13). The positive identification of these 18 isolates as belonging to *B. licheniformis* species was further strengthen by 16S rRNA gene sequencing. Consequently, the selected markers have proven reliable for DNA-based identification of *B. licheniformis* isolates. The consistency of these results is a promising alternative to the long ago established biochemical tests used to date for identification of *B. licheniformis* (Gordon et al., 1973).

The development of a reliable tool to monitor bacterial populations in fish faecal samples is critical to unveil gut microbiota patterns. qPCR studies of the fish microbiota are extremely recent and focused in gene expression analysis of the gut microbiome (Gerzova et al., 2014; Xia et al., 2014). On the other hand, the several reports about qPCR assays to target the human gut microorganisms are biased to well characterized gut bacteria such as bifidobacteria or other taxa well represented (Centanni et al., 2013; Furet et al., 2009; Haarman and Knol, 2005; Jost et al., 2012, 2014; Larsen et al., 2010; Mariat et al., 2009). Currently there is the urgency to improve accurate tools to assess and weigh up the dynamics of autochthonous and added bacteria in the gut microbiota. Having this into consideration, using *B. licheniformis* as the microorganism of interest and acknowledging the adequacy of the four *B. licheniformis* specific markers characterized in this work and described above, a validation procedure consisting in four key stages was put into practice, to reach a reliable qPCR method to quantify *B. licheniformis* in faecal samples of farmed fish microbiota. Those key stages were:

A. Markers' standard curve calibration for qPCR;
B. Determination of the LOQ;
C. Assessment of the DNA extraction method efficiency and determination of LOQ using faecal samples;
D. Quantification using DNA extracted from faecal samples.

A. Markers' standard curve calibration for qPCR

The BL8A and BL18A markers were selected for specific quantification of *B. licheniformis* in fish faecal samples through qPCR as these two markers provided amplicon sizes of 247 and 216 bp, respectively, and below the maximum size recommended for qPCR (250 bp), since decreased PCR efficiency is associated with longer products.

To construct standard curves for quantification, dilutions of plasmids carrying the target DNA have been previously reported with successful results (Menard et al., 2008). In the present work, we inserted the selected DNA markers BL8A and BL18A into pGEM-T plasmid obtaining the plasmids pEA1 and pEA3 respectively. Serial dilutions of both these plasmids were prepared and used to obtain the standard curves. The first assays for the standard curves calibration showed that all plasmid dilutions tested presented measurable Cqs with a high efficiency and robust $R^2$ for both markers (Fig. 15). Cq values are related with the quantification accuracy, since efficiency is calculated from the slope of the standard curve and represents the effectiveness of the PCR polymerase to duplicate the template DNA at each cycle. $R^2$ correlates the Cq values with the dilutions series, i.e. how well the points "fit the line". To obtain reliable quantification results, the values of efficiency must be between 90 and 110 % and $R^2$ equal or higher than 0.980 (Bio-Rad, 2013).

B. Determination of the LOQ

LOQ is defined as the lowest amount that can be quantified and is different from the limit of detection (LOD), which corresponds to the lowest amount detectable in a single reaction (Klaus, 1998). In qPCR, LOQ is determined as the amount of DNA corresponding to the Cq at which specificity and sensitivity of the experiment are maximized (Nutz et al., 2011). This parameter is extremely important to determine the optimal cut-off point above which the samples can be reliably quantified.

For this work, LOQ was determined using two alternative approaches: first, by estimating the mass of a *B. licheniformis* chromosome and using serial dilutions prepared from a known concentration of DNA extracted from *B. licheniformis*, it was possible to calculate LOQ between 1 and 70 chromosomes/µL. The second approach used DNA extracted from each of the serial dilutions of *B. licheniformis* cultures, for which the bacterial concentration was determined as CFUs/µL. This approach allowed to calculate LOQ between 1 and 8 CFU/µL, despite the efficiencies and $R^2$ values of these assays were slightly below to the reference values (efficiencies from 74.6 to 93.5

% and $R^2$ from 0.872 to 0.975). The difference between the LOQ values determined by these two approaches are likely the result of inevitable limitations, namely due to the presence of unviable or uncultured cells, but also due to the loss of DNA during the extraction procedure for each culture serial dilution, since DNA extraction efficiency depends on the method used (Lazarevic et al., 2013; McOrist et al., 2002).

C. Assessment of the DNA extraction method efficiency and determination of LOQ using faecal samples

Extraction of DNA from faecal samples has been associated with the co-extraction of PCR inhibitors (Alaeddini, 2012; Pontiroli et al., 2011; Schrader et al., 2012; Wilson, 1997), which can hamper bacterial quantification using qPCR. To determine if the DNA extraction method applied to faecal samples was efficient, and also to assess the cut-off point above which quantification of *B. licheniformis* in faecal samples can be reliable (LOQ), faecal samples were spiked with different concentrations of *B. licheniformis* strain 9945A cells: 0 (SPC0), 1 (SPC1), $10^3$ (SPC10^3) or $10^6$ (SPC10^6) CFUs/mg. Determination of LOQ is a common approach used in qPCR (Hein et al., 2001; Nutz et al., 2011) to increase the confidence of results.

Using the SPCs samples and using a DNA extraction procedure adapted from Pitcher et al. (1989), it was possible to determine LOQ value above $10^6$ CFUs/µL (Fig. 16). To further improve the resolution of the qPCR to quantify *B. licheniformis*, is important to narrow the LOQ value.

Not surprisingly, DNA extracted from pure cultures of *B. licheniformis*, using a standard commercial DNA extraction kit (EZNA Bacterial DNA Purification Kit, Omega Bio-Tek), presented much lower values of LOQ (1 to 70 chromosomes/µL when using DNA dilutions and 1 to 8 CFUs/µL when using DNA extracted from culture dilutions) comparing to those obtained with SPCs, for which LOQ value was set above $10^6$ CFUs/µL. Despite the DNA extraction procedure used in this work was previously optimized (data not shown) to ensure that the most suitable method to extract DNA from farmed fish faecal samples was used, this data suggest that DNA extraction methods for faecal samples still need to be improved to ensure higher efficiencies. Additional optimization of qPCR assays is also important to decrease the LOQ value obtained with faecal samples.

D. Quantification using DNA extracted from faecal samples

Beneficial bacteria of the gut microbiota have been associated with a favorable effect to the host. The extent of this relationship is however poorly understood. Although, qPCR has been recently used to study the gut microbiome (Gerzova et al., 2014; Xia et al., 2014), studies assessing the fish gut microbiota dynamics are still inexistent. This knowledge might be a powerful tool to understand the gut bacteria dynamics, as well as the effects of probiotics, prebiotics and synbiotics application in aquaculture production. These later findings will allow to select the adequate diet applied for each farm fish.

In the present study, we obtained faecal samples from gilthead seabream fed with diets differently enriched with FOS (0, 0.1, 0.25 and 0.5 % FOS). To assess the dynamics of *B. licheniformis* populations in these samples, several controls of the qPCR reaction were used, namely an IPC to screen for PCR inhibitors consisting in faecal sample 6 spiked with DNA from strain 9945A (IPC-6) and an unspecific amplification control consisting in faecal sample 6 spiked with DNA from *B. sonorensis* (Bsono-6), which is a closely related species to *B. licheniformis*. With these controls, we were able to determine the absence of PCR inhibitors and unspecific amplification in sample 6 (Fig. 17).

qPCR data obtained with the DNA extracted from faecal samples 1 to 12 was below the LOQ cut-off defined using SPCs, therefore, determination of the ability of FOS to modulate gut resident *B. licheniformis* was inconclusive (Fig. 18). It should be noted that, body parameters, such as size and weight, of seabream fishes used in this work were not affected when comparing the control group to fish fed with different levels of FOS (Guerreiro et al., NUTRIMU, unpublished results), meaning that it is possible that the FOS diets did not boost the *B. licheniformis* populations in the gut microbiota. Despite these results, it has been shown that FOS can modulate the gut microbiota, since FOS has been reported as an inducer of bacterial growth and activity of *Lactobacillus* and *Bifidobacterium* strains (Buddington et al., 1996; Gibson et al., 1995; Williams et al., 1994).

In conclusion, bioinformatics tools, such as CUPID and Insignia, have proven to be reliable to select novel taxa-specific markers. Markers obtained from these analyses were successfully applied for identification of *B. licheniformis* isolates by multiplex PCR. Additionally, the use of four *B. licheniformis* specific markers provided a reliable

approach for *B. licheniformis* detection on environmental samples surpassing the action of PCR inhibitors.

Finally, we were able to quantify *B. licheniformis* populations in environmental samples, even though additional optimization of the DNA extraction method and of the qPCR assay is required. Despite these limitations, the novel DNA markers specific for *B. licheniformis* characterized in the present work have proven to be reliable tools for culture-independent identification and detection of this species, allowing also the monitoring of *B. licheniformis* in environmental samples.

# Future Perspectives

In the present work, we selected and validated novel *B. licheniformis* specific DNA markers, which have proved to be reliable targets for DNA-based methodologies of identification, detection and monitoring of this bacterium.

In spite of these promising results and the fact that the markers were validated in several *B. licheniformis* isolates, extending these assays to a higher number of isolates from diverse ecological niches are needed to consolidate this approach as a reliable method for culture-independent identification of this bacteria species.

Concerning the detection and quantification of *B. licheniformis* on environmental samples by qPCR, the main challenge seems to be due to the co-extraction of inhibitors. As this limitation was shown to be biased for some markers and consequently primer pairs, efforts should be made to change qPCR conditions to an attempt to prevent unspecific primer annealing. In addition, improving DNA extraction may be a valuable help to increase qPCR efficiency, but more importantly increase the resolution of qPCR by reducing the LOQ. Once established, quantification of *B. licheniformis* by qPCR can provide a better understanding of its dynamics in gut microbiota of farmed fish in response to different diets, or to disease preventive measures, namely by the addition of probiotics, prebiotics and synbiotics, but also during disease outbreaks. This knowledge will certainly be a plus to optimize and implement best practices for aquaculture. Beyond the immediate interest to track and monitor *B. licheniformis* in gut microbiota of farm fish, which might lead to the discovery and characterization of new *B. licheniformis* probiotic strains, this work is ultimately a proof of concept that might be extended to other important farm fish gut bacteria, such as pathogens or gut resident bacteria with pertinent functional traits.

Finally, besides the applications described in the present work, one should keep in mind the utility of the taxa-specific markers for genotyping, i.e. to assess the clonal diversity of *B. licheniformis* in the fish gut microbiota.

# References

Agersø, Y., and Guardabassi, L. (2005). Identification of Tet 39, a novel class of tetracycline resistance determinant in *Acinetobacter* spp. of environmental and clinical origin. J. Antimicrob. Chemother. *55*, 566–569.

Alaeddini, R. (2012). Forensic implications of PCR inhibition--A review. Forensic Sci. Int. Genet. *6*, 297–305.

Albuquerque, P., Mendes, M. V., Santos, C.L., Moradas-Ferreira, P., and Tavares, F. (2009). DNA signature-based approaches for bacterial detection and identification. Sci. Total Environ. *407*, 3641–3651.

Albuquerque, P., Caridade, C.M.R., Marçal, A.R.S., Cruz, J., Cruz, L., Santos, C.L., Mendes, M. V, and Tavares, F. (2011). Identification of *Xanthomonas fragariae*, *Xanthomonas axonopodis* pv. *phaseoli*, and *Xanthomonas fuscans* subsp. *fuscans* with novel markers and using a dot blot platform coupled with automatic data analysis. Appl. Environ. Microbiol. *77*, 5619–5628.

Albuquerque, P., Caridade, C.M.R., Rodrigues, A.S., Marçal, A.R.S., Cruz, J., Cruz, L., Santos, C.L., Mendes, M. V., and Tavares, F. (2012a). Evolutionary and experimental assessment of novel markers for detection of *Xanthomonas euvesicatoria* in plant samples. PLoS ONE *7*, e37836.

Albuquerque, P., Rodrigues, A.S., Caridade, C.M.R., Marcal, A.R.S., and Tavares, F. (2012b). An in silico workflow for the selection of novel DNA markers for detection of phytopathogenic bacteria, using *Agrobacterium tumefaciens* as model. J. Plant Pathol. *94*, S1.83–S1.90.

Alderman, D.J., and Hastings, T.S. (1998). Antibiotic use in aquaculture: development of antibiotic resistance - potential for consumer health risks. Int. J. Food Sci. Technol. *33*, 139–155.

Almeida, A., Albuquerque, P., Araujo, R., Ribeiro, N., and Tavares, F. (2013). Detection and discrimination of common bovine mastitis-causing streptococci. Vet. Microbiol. *164*, 370–377.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic Local Alignment Search Tool. J. Mol. Biol. *215*, 403–410.

Amann, R. (2000). Who is out there? Microbial aspects of biodiversity. Syst. Appl. Microbiol. *23*, 1–8.

Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. *59*, 143–169.

Angulo, F. (2000). Antimicrobial agents in aquaculture: potential impact on public health. APUA Newsl. *18*, 1–5.

Angulo, F.J., Nargund, V.N., and Chiller, T.C. (2004). Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance. J. Vet. Med. *51*, 374–379.

Araya, M., Morelli, L., Reid, G., and Sanders, M.E. (2002). Guidelines for the evaluation of probiotics in food - Joint FAO/WHO Working Group (Ontario, Canada).

Avella, M.A., Gioacchini, G., Decamp, O., Makridis, P., Bracciatelli, C., and Carnevali, O. (2010). Application of multi-species of *Bacillus* in sea bream larviculture. Aquaculture *305*, 12–19.

Bagheri, T., Hedayati, S.A., Yavari, V., Alizade, M., and Farzanfar, A. (2008). Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. Turkish J. Fish. Aquat. Sci. *8*, 43–48.

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., and Múzquiz, J.L. (2006). The role of probiotics in aquaculture. Vet. Microbiol. *114*, 173–186.

Barbosa, T.M., Serra, C.R., Ragione, R.M. La, Woodward, M.J., and Henriques, A.O. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. *71*, 968–978.

Barrangou, R., Altermann, E., Hutkins, R., Cano, R., and Klaenhammer, T.R. (2003). Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc. Natl. Acad. Sci. U. S. A. *100*, 8957–8962.

Barton, B.A., and Ywama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 3–26.

Bio-Rad (2013). Reagent comparison guide for real-time PCR.

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. Eur. J. Nutr. *41 Suppl 1*, I11–I16.

Buddington, K.K., Donahoo, J.B., and Buddington, R.K. (2002). Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. J. Nutr. *132*, 472–477.

Buddington, R.K., Williams, C.H., Chen, S.-C., and Witherly, S.A. (1996). Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. Am. J. Clin. Nutr. *63*, 709–716.

Burr, G., Gatlin III, D., and Ricke, S. (2005). Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. J. World Aquac. Soc. *36*, 425–436.

Casas, C., Anderson, E.C., Ojo, K.K., Keith, I., Whelan, D., Rainnie, D., and Roberts, M.C. (2005). Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. FEMS Microbiol. Lett. *242*, 59–63.

Centanni, M., Turroni, S., Biagi, E., Severgnini, M., Consolandi, C., Brigidi, P., and Candela, M. (2013). A novel combined approach based on HTF-Microbi.Array and qPCR for a reliable characterization of the *Bifidobacterium*-dominated gut microbiota of breast-fed infants. FEMS Microbiol. Lett. *343*, 121–126.

Claus, D., and Berkeley, R.C.W. (1986). Genus *Bacillus* Cohn 1872. In Bergey's Manual of Systematic Bacteriology, Vol 2, P.H.A. Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt., eds. (Baltimore, Maryland: Williams and Wilkins Co.), pp. 1105–1139.

Clemente, J.C., Ursell, L.K., Parfrey, L.W., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. Cell *148*, 1258–1270.

Collins, M.D., and Gibson, G.R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nutr. *69*, 1052S–1057S.

Cunningham, C.O. (2002). Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture *206*, 19–55.

Cutting, S.M. (2011). *Bacillus* probiotics. Food Microbiol. *28*, 214–220.

Dalmin, G., Kathiresan, K., and Purushothaman, A. (2001). Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. Indian J. Exp. Biol. *39*, 939–942.

Decamp, O., Moriarty, D.J.W., and Lavens, P. (2008). Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac. Res. *39*, 334–338.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., and Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol. *25*, 472–479.

DGRM (2014). Aquicultura.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Güroy, D., and Davies, S.J. (2011). Microbial manipulations to improve fish health and production – A Mediterranean perspective. Fish Shellfish Immunol. *30*, 1–16.

Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O., and Cutting, S.M. (2004). Characterization of *Bacillus* probiotics available for human use. Appl. Environ. Microbiol. *70*, 2161–2171.

EC (2014). European Commission - Aquaculture.

FAO (2014a). State of the world's fisheries and aquaculture (Rome, Italy).

FAO (2014b). Impacts of fishery activities.

FAO (2014c). Aquaculture - FAO's role in aquaculture.

FAO (2014d). Aquaculture.

FAO (2014e). FOCUS - The challenge of sustainable production.

FAO (2014f). FOCUS - Sustainable aquaculture development.

FAO (2014g). About FAO - Who we are.

FAO (2014h). Cultured aquatic species information programme - *Sparus aurata* (Linnaeus, 1758).

FAO (2014i). Species fact sheets - *Sparus aurata* (Linnaeus, 1758).

FAO (2014j). Aquactic species distribution map viewer.

FishBase (2014). *Sparus aurata* Linnaeus, 1758 - Gilthead seabream.

Framian, B. (2009). Definition of data collection needs for aquaculture - Review of the EU aquaculture sector and results of costs and earnings survey.

Fuhrman, J.A. (1999). Marine viruses and their biogeochemical and ecological effects. Nature *399*, 541–548.

Furet, J.-P., Firmesse, O., Gourmelon, M., Bridonneau, C., Tap, J., Mondot, S., Doré, J., and Corthier, G. (2009). Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol. Ecol. *68*, 351–362.

Gerzova, L., Videnska, P., Faldynova, M., Sedlar, K., Provaznik, I., Cizek, A., and Rychlik, I. (2014). Characterization of microbiota composition and presence of selected antibiotic resistance genes in carriage water of ornamental fish. PLoS ONE *9*, e103865.

Ghosh, K., Sen, S.K., and Ray, A.K. (2002). Characterization of bacilli isolated from the gut of rohu, *Labeo rohita*, fingerlings and its significance in digestion. J. Appl. Aquac. *12*, 33–42.

Gibson, G.R., and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. *125*, 1401–1412.

Gibson, G.R., Beatty, E.R., Wang, X.W., and Cummings, J.H. (1995). Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology *108*, 975–982.

Goh, Y.J., Zhang, C., Benson, A.K., Schlegel, V., Lee, J.-H., and Hutkins, R.W. (2006). Identification of a putative operon involved in fructooligosaccharide utilization by *Lactobacillus paracasei*. Appl. Environ. Microbiol. *72*, 7518–7530.

Goh, Y.J., Lee, J.-H., and Hutkins, R.W. (2007). Functional analysis of the fructooligosaccharide utilization operon in *Lactobacillus paracasei* 1195. Appl. Environ. Microbiol. *73*, 5716–5724.

Goldburg, R.J., Elliott, M.S., and Naylor, R.L. (2001). Marine aquaculture in the United States: environmental impacts and policy options. Pew Ocean. Comm. 1–28.

Gómez-Doñate, M., Ballesté, E., Muniesa, M., and Blanch, A.R. (2012). New molecular quantitative PCR assay for detection of host-specific Bifidobacteriaceae suitable for microbial source tracking. Appl. Environ. Microbiol. *78*, 5788–5795.

Gordon, R.H.W.C., Haynes, W., and Pang, C. (1973). The genus *Bacillus*. In Agriculture Handbook, (Washington, D.C.: US Department of Agriculture), pp. 109–126.

Grave, K., Markestad, A., and Bangen, M. (1996). Comparison in prescribing patterns of antibacterial drugs in salmonid farming in Norway during the periods 1980-1988 and 1989-1994. J. Vet. Pharmacol. Ther. *19*, 184–191.

Grave, K., Lingaas, E., Bangen, M., and Rønning, M. (1999). Surveillance of the overall consumption of antibacterial drugs in humans, domestic animals and farmed fish in Norway in 1992 and 1996. J. Antimicrob. Chemother. *43*, 243–252.

Guerreiro, I. (2013). Prebiotics use in *Sparus aurata* and *Scophthalmus maximus production* - First year repor, doctoral Program in Biology.

Haarman, M., and Knol, J. (2005). Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. Appl. Environ. Microbiol. *75*, 2318–2324.

Hartemink, R., Quataert, M.C.J., van Laere, K.M.J., Nout, M.J.R., and Rombouts, F.M. (1995). Degradation and fermentation of fructo-oligosaccharides by oral streptococci. J. Appl. Bacteriol. *79*, 551–557.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., and Wagner, M. (2001). Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol. *67*, 3122–3126.

Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., and Cutting, A.S. (2000). Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. *66*, 5241–5247.

Huys, G., Rhodes, G., McGann, P., Denys, R., Pickup, R., Hiney, M., Smith, P., and Swings, J. (2000). Characterization of oxytetracycline-resistant heterotrophic bacteria originating from hospital and freshwater fishfarm environments in England and Ireland. Syst. Appl. Microbiol. *23*, 599–606.

ISME - International Society for Microbial Ecology (2013). What is microbial ecology?

Jost, T., Lacroix, C., Braegger, C.P., and Chassard, C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. PLoS ONE *7*, e44595.

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2014). Stability of the maternal gut microbiota during late pregnancy and early lactation. Curr. Microbiol. *68*, 419–427.

Klaus, R. (1998). Detection limits and quantitation limits in the view of international harmonization and the consequences for analytical laboratories. Accredit. Qual. Assur. *3*, 150–154.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics, E. Stackebrandt, and M. Goodfellow, eds. (New York, USA: John Wiley & Sons Ltd.), p. 115.175.

Larsen, N., Vogensen, F.K., van den Berg, F.W.J., Nielsen, D.S., Andreasen, A.S., Pedersen, B.K., Al-Soud, W.A., Sørensen, S.J., Hansen, L.H., and Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS ONE *5*, e9085.

Lazarevic, V., Gaïa, N., Girard, M., François, P., and Schrenzel, J. (2013). Comparison of DNA extraction methods in analysis of salivary bacterial communities. PLoS ONE *8*, e67699.

Lee, Z.M.-P., Bussema, C., and Schmidt, T.M. (2008). rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res. *37*, D489–D493.

Li, K., Zheng, T., Tian, Y., Xi, F., Yuan, J., Zhang, G., and Hong, H. (2007). Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*. Biotechnol. Lett. *29*, 525–530.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature *489*, 220–230.

Lyons, E., and Freeling, M. (2008). How to usefully compare homologous plant genes and chromosomes as DNA sequences. Plant J. *53*, 661–673.

Lyons, E., Pedersen, B., Kane, J., and Freeling, M. (2008). The value of nonmodel genomes and an example using SynMap within CoGe to dissect the hexaploidy that predates the rosids. Trop. Plant Biol. *1*, 181–190.

Madslien, E.H., Olsen, J.S., Granum, P.E., and Blatny, J.M. (2012). Genotyping of *B. licheniformis* based on a novel multi-locus sequence typing (MLST) scheme. BMC Microbiol. *12*, 230.

Mahious, A.S., Gatesoupe, F.J., Hervi, M., Metailler, R., and Ollevier, F. (2006). Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). Aquac. Int. *14*, 219–229.

Mariat, D., Firmesse, O., Levenez, F., Guimarães, V.D., Sokol, H., Doré, J., Corthier, G., and Furet, J.-P. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. *9*, 123.

Martinsen, B., and Horsberg, T.E. (1995). Comparative single-dose pharmacokinetics of four quinolones, oxolinic acid, flumequine, sarafloxacin, and enrofloxacin, in Atlantic salmon (*Salmo salar*) held in seawater at 10 degrees C. Antimicrob. Agents Chemother. *39*, 1059–1064.

Mazumder, R., Natale, D.A., Murthy, S., Thiagarajan, R., and Wu, C.H. (2005). Computational identification of strain-, species- and genus-specific proteins. BMC Bioinformatics *6*, 279.

Mckellar, R.C., and Modler, H.W. (1989). Metabolism of fructo-oligosaccharides by *Bifidobacterium* spp. Appl. Microbiol. Biotechnol. *31*, 537–541.

McOrist, A.L., Jackson, M., and Bird, A.R. (2002). A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods *50*, 131–139.

Menard, J.-P., Fenollar, F., Henry, M., Bretelle, F., and Raoult, D. (2008). Molecular quantification of Gardnerella *vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. Clin. Infect. Dis. *47*, 33–43.

Menéndez, C., Hernández, L., Selman, G., Mendoza, M.F., Hevia, P., Sotolongo, M., and Arrieta, J.G. (2002). Molecular cloning and expression in *Escherichia coli* of an exo-levanase gene from the endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4. Curr. Microbiol. *45*, 5–12.

Metchnikoff, É. (1907). Lactic acid as inhibiting intestinal putrefaction. In The Prolongation of Life: Optimistic Studies, (London: Heinemann), pp. 161–183.

Michon, A.-L., Aujoulat, F., Roudière, L., Soulier, O., Zorgniotti, I., Jumas-Bilak, E., and Marchandin, H. (2010). Intragenomic and intraspecific heterogeneity in rrs may surpass interspecific variability in a natural population of *Veillonella*. Microbiology *156*, 2080–2091.

Miranda, C.D., and Zemelman, R. (2002a). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture *212*, 31–47.

Miranda, C.D., and Zemelman, R. (2002b). Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. Sci. Total Environ. *293*, 207–218.

Moriarty, D.J.W. (1998). Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture *164*, 351–358.

Moriarty, D.J.W. (2003). Probiotics in aquaculture. Microbiol. Aust. *March*, 15–17.

Nagano, H., and To, K.A. (1999). Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. Biosci. Biotechnol. Biochem. *63*, 181–183.

Naumoff, D.G. (2001). Beta-fructosidase superfamily: homology with some alpha-L-arabinases and beta-D-xylosidases. Proteins *42*, 66–76.

Naylor, R., and Burke, M. (2005). Aquaculture and ocean resources: raising tigers of the sea. Annu. Rev. Environ. Resour. *30*, 185–218.

Nutz, S., Döll, K., and Karlovsky, P. (2011). Determination of the LOQ in real-time PCR by receiver operating characteristic curve analysis: application to qPCR assays for *Fusarium verticillioides* and *F. proliferatum*. Anal. Bioanal. Chem. *401*, 717–726.

Phillippy, A.M., Mason, J. a, Ayanbule, K., Sommer, D.D., Taviani, E., Huq, A., Colwell, R.R., Knight, I.T., and Salzberg, S.L. (2007). Comprehensive DNA signature discovery and validation. PLoS Comput. Biol. *3*, e98.

Phillippy, A.M., Ayanbule, K., Edwards, N.J., and Salzberg, S.L. (2009). Insignia: a DNA signature search web server for diagnostic assay development. Nucleic Acids Res. *37*, W229–W234.

Pitcher, D.G., Saunders, N.A., and Owen, R.J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. *8*, 151–156.

Pontiroli, A., Travis, E.R., Sweeney, F.P., Porter, D., Gaze, W.H., Mason, S., Hibberd, V., Holden, J., Courtenay, O., and Wellington, E.M.H. (2011). Pathogen quantitation in complex matrices: a multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. PLoS ONE *6*, e17916.

Prosser, J.I., Bohannan, B.J.M., Curtis, T.P., Ellis, R.J., Firestone, M.K., Freckleton, R.P., Green, J.L., Green, L.E., Killham, K., Lennon, J.J., et al. (2007). The role of ecological theory in microbial ecology. Nat. Rev. Microbiol. *5*, 384–392.

Puigbò, P., Bravo, I.G., and Garcia-Vallve, S. (2008). CAIcal: a combined set of tools to assess codon usage adaptation. Biol. Direct *3*, 38.

Raida, M.K., Larsen, J.L., Nielsen, M.E., and Buchmann, K. (2003). Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). J. Fish Dis. *26*, 495–498.

Rastall, R.A., and Maitin, V. (2002). Prebiotics and synbiotics: towards the next generation. Curr. Opin. Biotechnol. *13*, 490–496.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., and Menasveta, P. (1998). Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture *167*, 301–313.

Rengpipat, S., Tunyanun, A., Fast, A.W., Piyatiratitivorakul, S., and Menasveta, P. (2003). Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. Dis. Aquat. Organ. *55*, 169–173.

Ringø, E., Olsen, R.E., Gifstad, T.Ø., Dalmo, R.A., Amlund, H., Hemre, G.-I., and Bakke, A.M. (2010). Prebiotics in aquaculture: a review. Aquac. Nutr. *16*, 117–136.

Romero, J., Feijoó, C.G., and Navarrete, P. (2012). Antibiotics in aquaculture – use, abuse and alternatives. In Health and Environment in Aquaculture, E. Carvalho, ed. (Santiago: InTech Publisher), pp. 159–198.

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., and Matteuzzi, D. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl. Environ. Microbiol. *71*, 6150–6158.

Saito, K., Kondo, K., Kojima, I., Yokota, A., and Tomita, F. (2000). Purification and characterization of 2,6-beta-D-fructan 6-levanbiohydrolase from *Streptomyces exfoliatus* F3-2. Appl. Environ. Microbiol. *66*, 252–256.

Salinas, I., Cuesta, A., Esteban, M.A., and Meseguer, J. (2005). Dietary administration of *Lactobacillus delbrüeckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish Shellfish Immunol. *19*, 67–77.

Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., and Esteban, M.A. (2008). Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. *25*, 114–123.

Samuelsen, O.B. (2006). Pharmacokinetics of quinolones in fish: a review. Aquaculture *255*, 55–75.

Saulnier, D.M.A., Molenaar, D., de Vos, W.M., Gibson, G.R., and Kolida, S. (2007). Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl. Environ. Microbiol. *73*, 1753–1765.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K., and Larsen, J.L. (2000). Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four danish rainbow trout farms. Appl. Environ. Microbiol. *66*, 4908–4915.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., and Larsen, J.L. (2001a). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol. *67*, 5675–5682.

Schmidt, A.S., Bruun, M.S., Larsen, J.L., and Dalsgaard, I. (2001b). Characterization of class 1 integrons associated with R-plasmids in clinical *Aeromonas salmonicida* isolates from various geographical areas. J. Antimicrob. Chemother. *47*, 735–743.

Schrader, C., Schielke, A., Ellerbroek, L., and Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. *113*, 1014–1026.

SciencePrimer (2013). Copy number calculator for realtime PCR.

Smith, P., and Samuelsen, O.B. (1996). Estimates of the significance of out-washing of oxytetracycline from sediments under Atlantic salmon sea-cages. Aquaculture *144*, 17–26.

Song, E.-K., Kim, H., Sung, H.-K., and Cha, J. (2002). Cloning and characterization of a levanbiohydrolase from *Microbacterium laevaniformans* ATCC 15953. Gene *291*, 45–55.

Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., and Oggioni, M.R. (2000). On the fate of ingested *Bacillus* spores. Res. Microbiol. *151*, 361–368.

Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques *28*, 1102–1104.

Sun, Y., Wen, Z., Li, X., Meng, N., Mi, R., Li, Y., and Li, S. (2011). Dietary supplement of fructooligosaccharides and *Bacillus subtilis* enhances the growth rate and disease resistance of the sea cucumber *Apostichopus japonicus* (Selenka). Aquac. Res. *43*, 1328–1334.

Tapia-Paniagua, S.T., Chabrillón, M., Díaz-Rosales, P., de la Banda, I.G., Lobo, C., Balebona, M.C., and Moriñigo, M.A. (2010). Intestinal microbiota diversity of the flat fish *Solea senegalensis* (Kaup, 1858) following probiotic administration. Microb. Ecol. *60*, 310–319.

Vaseeharan, B., and Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Appl. Microbiol. *36*, 83–87.

Verschuere, L., Rombaut, G., and Sorgeloos, P. (2000). Probiotic bacteria as biological control agents in aquaculture probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. *64*, 655–671.

De Vrese, M., and Schrezenmeir, J. (2008). Probiotics, prebiotics, and synbiotics. Adv. Biochem. Eng. Biotechnol. *111*, 1–66.

Walker, P.J. (2004). Disease emergence and food security: global impact of pathogens on sustainable aquaculture production. In Fish, Aquaculture and Food Security, A.G. Brown, ed. (Canberra, Australia: The ASTE Crawford Fund), pp. 44–50.

Wang, Y.-B., Li, J.-R., and Lin, J. (2008). Probiotics in aquaculture: challenges and outlook. Aquaculture *281*, 1–4.

Weber, J.T., Mintz, E.D., Cañizares, R., Semiglia, A., Gomez, I., Sempértegui, R., Dávila, A., Greene, K.D., Puhr, N.D., and Cameron, D.N. (1994). Epidemic cholera in Ecuador: multidrug-resistance and transmission by water and seafood. Epidemiol. Infect. *112*, 1–11.

Williams, C.H., Witherly, S.A., and Buddington, R.K. (1994). Influence of dietary neosugar on selected bacterial groups of the human faecal microbiota. Microb. Ecol. Health Dis. *7*, 91–97.

Wilson, I.G. (1997). Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. *63*, 3741–3751.

Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J., and Yue, G.H. (2014). The intestinal microbiome of fish under starvation. BMC Genomics *15*, 266.

Xiao, R., Tanida, M., and Takao, S. (1989). Purification and characteristics of two exoinulinases from *Chrysosporium pannorum*. J. Ferment. Bioeng. *67*, 331–334.

Zhang, C.-N., Li, X.-F., Xu, W.-N., Jiang, G.-Z., Lu, K.-L., Wang, L.-N., and Liu, W.-B. (2013). Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant capability and disease resistance of triangular bream (*Megalobrama terminalis*). Fish Shellfish Immunol. *35*, 1380–1386.

Zhang, Q., Ma, H., Mai, K., Zhang, W., Liufu, Z., and Xu, W. (2010). Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, non-specific immunity of sea cucumber, *Apostichopus japonicus*. Fish Shellfish Immunol. *29*, 204–211.

Ziaei-Nejad, S., Rezaei, M.H., Takami, G.A., Lovett, D.L., Mirvaghefi, A.-R., and Shakouri, M. (2006). The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture *252*, 516–524.

# Attachments

**Attachment I –** 16S rRNA sequences of non-*B. licheniformis* isolates.

**FI38 – 16S rRNA**

TGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGG
TTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTG
CTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAG
CCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCC
ACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCT
TCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCACCTCCTGTCCTGAAATGTTGGGTTAAGTCCCC
CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGG

**FI39 – 16S rRNA**

GCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA
CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGT
TCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGC
TTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCA
CGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT
GACGCTGAAGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG

AGTGCTAAGTGTTAAGGGGGTTTCCGCCCCCTTATTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
GAGTACGGTCGCAAGACTGAAACTCCAA

**FI46 – 16S rRNA**

GCAGTCGAGCGGACTTAAAAAGCTTGCTTTTTAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACC
TGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATGCTTTTTGACACATGTCGG
AAAGCTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGTACCGTAGTAACTGCCG
GTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGA
CGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACTTTCCCCTTC
GGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG

**FI157 – 16S rRNA**

ATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTG
GGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAGACATAAAAGGTG
GCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
GACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
TTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGT
ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGG
GGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAG
GGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACATCCTAGAGATAGGACGTCCCCTTCGGGGGCAAAA

**Attachment II –** 16S rRNA sequences of *B. licheniformis* isolates.

**FI42 – 16S rRNA**

TGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGG
TTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAAACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAACAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCACAGCCGCGGTAAACGTAG
GTGGCAGCGTTGCCGGAATTATTGGGCGTAAGCGCGCGCAGGCGGTTTTCTTAAGTCTGATGTGAAAGCC
CCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCAC
GTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTC
GGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
GCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT

**FI44 – 16S rRNA**

TGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGG
TTCAATCATAAAAGGGGGCTTTTAACTACCACTTACAGATGGACCCCCGGCGCATTAACTAGTTGGTGAG
GTAACGGCTCACCAAGGGGACCATGCGTAACCCAACTGAAAGGGTGATCGGCCACCCTGGGACTGAAACA
CCGCCCAAACTCCTACGGGAAGCAACAATAAGGAATCTTCCCCAATGGAAGAAAGTCTGACGGAACAACG
CCCCGTGAGTGATGAAAGGTTTCGGATCGTAAAACTCTGTTGTTAGGGAAAAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACCGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAA
AGCCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGG
GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCC

CCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC
AATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAA
CCTTTTGGAGCCAGCCGC

**FI47 – 16S rRNA**

TGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGG
TTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAACAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAA
AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCC
CTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA
ATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATC
GCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC
CTTTTGAGCCAGCC

**FI152 – 16S rRNA**

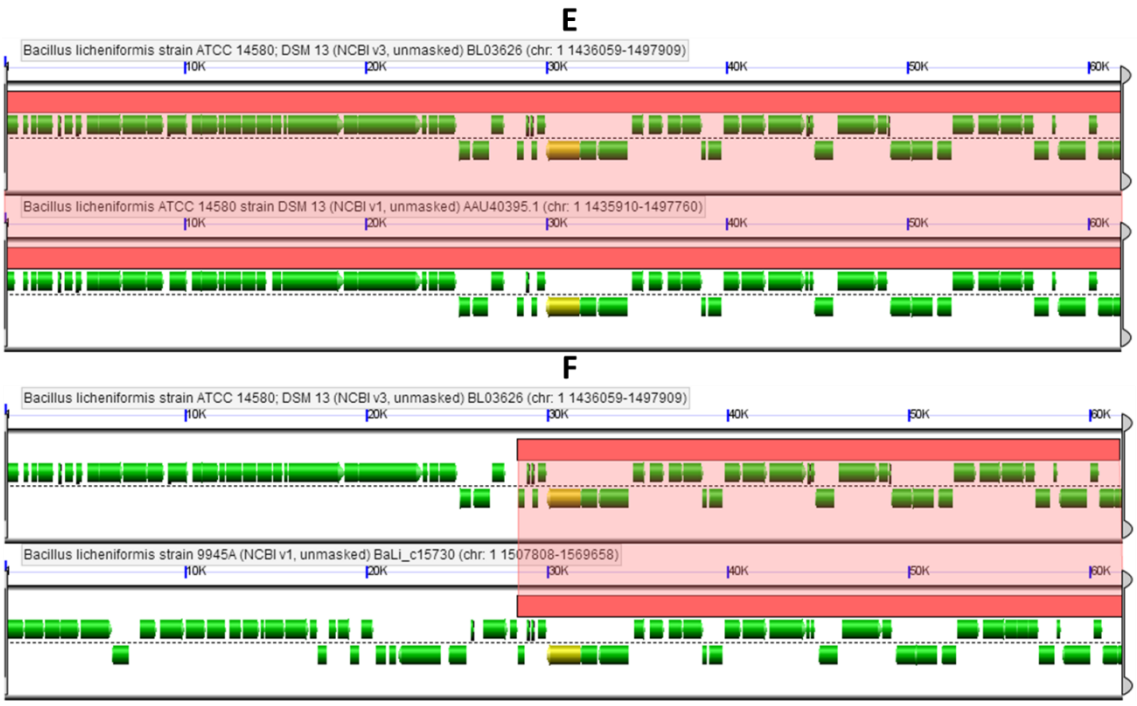
TGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGG
TTCAATCATAAAAGGTGGCTTTTAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT

AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAA
AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCC
CTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA
ATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATC
GCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC
CTTTTGGAGCCAGCCGCCGAA

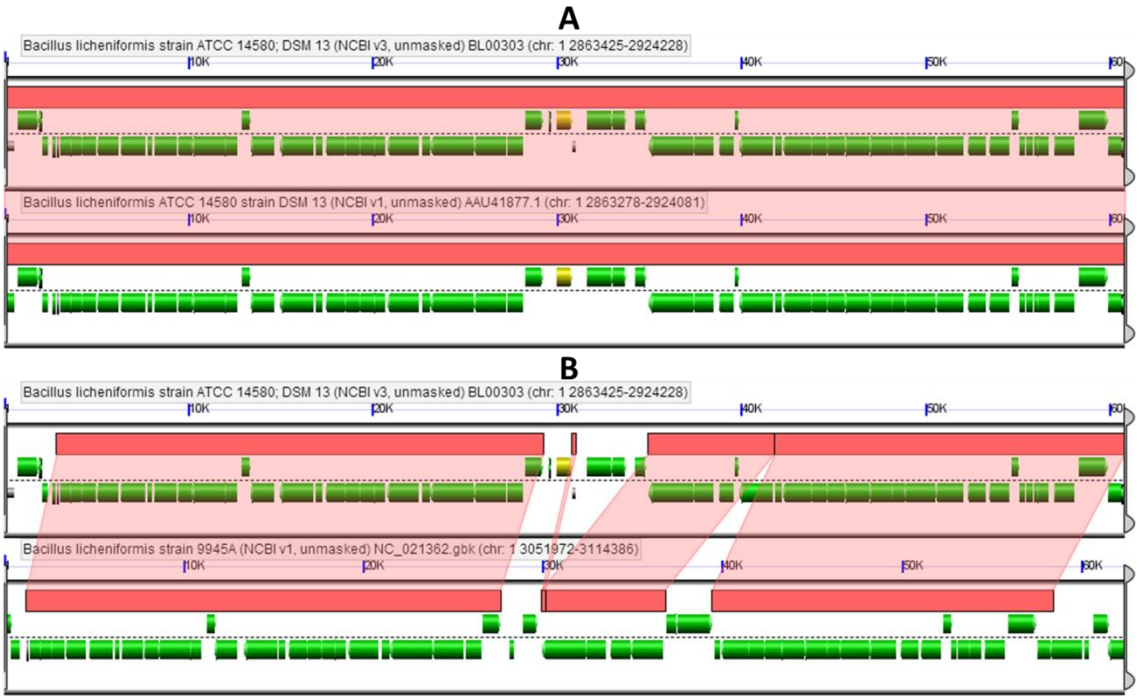
**FI159 – 16S rRNA**

TGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTA
ACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGG
TTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAACAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAA
AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCC
CTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA
ATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATC
GCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC
CTTT

**Attachment III –** High resolution synteny analysis comparing *B. licheniformis* ATCC_14580 with *B. licheniformis* DSM_13 (A, C and E) or with *B. licheniformis* 9945A (B, D and F). A and B – *serA2* (BL8A); C and D – BLi00806 (BL13C); E and F – *ligD* (BL18A). The ORFs containing DNA markers are highlighted in yellow. Areas colored in pink represent syntenic genomic regions.


E
Bacillus licheniformis strain ATCC 14580; DSM 13 (NCBI v3, unmasked) BL03626 (chr: 1 1436059-1497909)
Bacillus licheniformis ATCC 14580 strain DSM 13 (NCBI v1, unmasked) AAU40395.1 (chr: 1 1435910-1497760)
F
Bacillus licheniformis strain ATCC 14580; DSM 13 (NCBI v3, unmasked) BL03626 (chr: 1 1436059-1497909)
Bacillus licheniformis strain 9945A (NCBI v1, unmasked) BaLi_c15730 (chr: 1 1507808-1569658)

**Attachment IV –** High resolution synteny analysis of BL00303 (BL5B) region comparing *B. licheniformis* ATCC_14580 with *B. licheniformis* DSM_13 (A) or with *B. licheniformis* 9945A (B). The ORF containing BL5B marker is highlighted in yellow. Areas colored in pink represent syntenic genomic regions.



**Attachment V –** Sequences of the DNA markers specific for *B. licheniformis* used in this work, which were amplified using isolate FI1 as template. Primers used to amplify each marker are underlined.

**BL5B** (332 bp)

CGCTCACCATATGCACAGCTCTGGCATTTTCTCTCTTTATTTTCTTCAACAAGGCGAACTTTACAGAGGC
CAGCGAAAATAAAGAAACCGGGCTTTTCTTTGCCCGAAACTACATCAGCCATATGATGGAAAACGAGACG
ATGTCGTTTAACATTTTCGGCCTGCAAAAAGCAAACGCCGGCGTTCCTCTAAGCGGTGAGACGGTTACGT
CGCTCGCTTTTGATAACAATCATATACAAATCAGCGATTACAAGGTTGAAACAGGGATTCGCCATAAAGG
CTATACGCTCGTCAACATCATCGTAGATGTCCGAGTCTCAAGCGATAAACCG

**BL8A** (247 bp)

TCACAACCCGTTGACGACAAACTCCGGTTTTTGTTTGTCGATCAGTACACTAGCGATGTTTTTGACGCAT
ATCATGCCAAGGTTCTGTACGGCATCTCGGGTATACCCCGCGATATGCGGCACGGCGATAAAACGGTCCA
GCTCAAAGAGAGGGTGCTGCTTCAGCGGTTCGGTTTGATACACGTCAAGCGCAGCTCCAGCGATGCCCCC
GCTCGCCAAGGCATCATATAACGCACACTCGGACACG

**BL13C** (376 bp)

TTGTGCGTATCTCCGGGCCATTCTCCAAAAGGTTTTCCCGATGCTGAAATATTTCAAGGACTGTCTTTGA
TTTTCCTCAAGCTCCTTCCATTTGATATGAGGAAAATAGACGCTTTCGTCAAATTCTTTCACAATATAAT
CCATGACGTACACAAGCGATTGAATTCTTTTATGCAATTGGTAGGAAATGTTTTTTTGGTGGCGGCGGTA
GCATAATACAGGTTTTTTTAAATAGCGCCGCTTCCAGCCGCGCTTCGTATTGGCTAAACAATTCAGCACA
TCCCCTGCAAGCGGCTTTTGCGGATCAACCAGCCAAGTGAAGCCTTTTTGCTGATAATAAGATGCTTTAA
ACATGCCCACCATCGGGACAATGCCT

**BL18A** (216 bp)

GTCAACGACACAATTTCCCCGTCAATCGTGATCGGCAAGTGTTCTTTGAGAGTTTCGATCATGCTTTTTG
CAAATTCCGAAATTTCCGGAAATGTGTTTTCGAGCGGCTGTGAGTTTCTGCTGATGAGCGAAACTTCGCC
GGTCGCAGCAATCTTTAAAAGACCCCGATACCCGTCATATTTGACTTCATACCGCCAATTGCCGCCTGAG
GGAGCT